

## Spread and interaction of *Pepino mosaic virus* (PepMV) and *Pythium aphanidermatum* in a closed nutrient solution recirculation system: effects on tomato growth and yield

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*Pepino mosaic virus* (PepMV) was shown to be efficiently transmitted between tomato plants grown in a closed recirculating hydroponic system. PepMV was detected in all plant parts after transmission via contaminated nutrient solution using ELISA, immunocapture RT-PCR, RT-PCR, electron microscopy, and by inoculation to indicator plants. Detection of PepMV in nutrient solution was only possible after concentration by ultracentrifugation followed by RT-PCR. Roots tested positive for PepMV 1–3 weeks after inoculation, and subsequently a rapid spread from the roots into the young leaves and developing fruits was found within 1 week. PepMV was only occasionally detected in the older leaves. None of the infected plants showed any symptoms on fruits, leaves or other organs. Pre-infection of roots of tomato cv. Hildares with *Pythium aphanidermatum* significantly delayed PepMV root infections. When mechanically inoculated with PepMV at the 2–4 leaf stage, yield loss was observed in all plants. However, only plants of cv. Castle Rock recorded significant yield losses when infected via contaminated nutrient solution. Yield losses induced by infection with PepMV and/or *P. aphanidermatum* ranged from 0.4 up to 40% depending on experimental conditions.

**Keywords:** epidemiology, hydroponics, *Lycopersicon esculentum*, plant-pathogen interaction, *Solanum lycopersicum*, water transmission

### Introduction

The importance of soilless horticultural production using open or closed hydroponic systems has increased worldwide during the last three decades, particularly in tomato production (Savvas, 2003). In these production systems, plants are usually supplied with a nutrient solution circulating in gullies to allow more accurate control of the root environment. This can result in optimal use of water and nutrients, and thus in higher yields and better fruit quality. However, recirculating the nutrient solution facilitates the rapid and efficient spread of root-infecting pathogens throughout the whole crop and may thus increase the risk of epidemics when not managed well (Stanghellini & Rasmussen, 1994a). More than 20 fungal pathogens and a smaller number of viral and bacterial pathogens have been identified as causal agents of root

diseases in hydroponically grown crops. Some have been associated with extensive crop losses and there is evidence that the introduction and spread of plant pathogens by irrigation water is a significant epidemiological factor (Stanghellini & Rasmussen, 1994b; Hong & Moorman, 2005).

*Pythium aphanidermatum* is a major recurring problem in horticultural crops because of its aggressiveness and broad host range. It is responsible for considerable losses in tomato and other crops grown on artificial substrates in closed soilless systems, such as cucumber, pepper, lettuce and spinach (Sutton *et al.*, 2006). Root infection by *Pythium* spp. may reduce yield of plants through the decay of root tissue, but yield losses can also occur in the absence of severe root necrosis, as infection reduces water uptake and may cause leaves or shoots to wilt during warm temperatures (Favrin *et al.*, 1988).

Some of the pathogenic viruses transmitted through nutrient solution utilize fungal vectors belonging to the Chytridiomycetes (Campbell, 1996). For example, *Olpidium* spp. were shown to transmit *Mirafiori lettuce*

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*big-vein virus* in lettuce, *Tobacco necrosis virus* in bean and cucumber, and *Cucumber necrosis virus*, *Cucumber leaf spot virus* and *Melon necrotic spot virus* in cucumber (Paludan, 1985; Stanghellini & Rasmussen, 1994b; Campbell, 1996; Navarro *et al.*, 2005). Other plant viruses spread in hydroponic systems without the help of fungal vectors, e.g. *Cucumber green mottle mosaic virus*, *Cucumber mosaic virus* and *Arabidopsis mosaic virus* infecting cucumber (Paludan, 1985; Büttner *et al.*, 1995), *Tomato mosaic virus* (ToMV) and *Tobacco mosaic virus* (TMV) infecting pepper and tomato (Paludan, 1985; Pares *et al.*, 1992; Büttner *et al.*, 1995; Park *et al.*, 1999), and some viruses of ornamental plants (Krczal *et al.*, 1995). Vector-independent transmission of ToMV and TMV through the recirculating nutrient solution has been demonstrated, but both viruses are also mechanically transmitted by leaf contact and cultural operations, and the relative epidemiological relevance of the different transmission pathways is not clear.

Since 1999, *Pepino mosaic virus* (PepMV) has attracted much attention because it is found widely in tomato greenhouses in many European countries, Morocco, South and North America, and China (see references in Spence *et al.*, 2006). The origin of its sudden occurrence is not clear. However, similar to TMV and ToMV, rapid transmission and spread of PepMV within and between greenhouses can be facilitated mechanically by tools, clothes and the hands of workers contaminated during crop handling (Jones *et al.*, 1980).

Typical symptoms of infected leaves are rolling, light-yellow mosaics, dark-green discoloration and leaf distortion (Jordá *et al.*, 2001). Fruits may show yellow blotches, necrotic or yellow spots and irregular ripening (French *et al.*, 2000). Symptom expression can be affected by the tomato cultivar, the genotype of the virus, and the environmental conditions (French *et al.*, 2000; Lesemann, 2002; Hanssen *et al.*, 2009). There are conflicting reports on yield losses caused by PepMV infection. While Soler-Aleixandre *et al.* (2005) reported high losses with the collapse of up to 90% of plants, others described low yield losses of up to 15% (Verhoeven *et al.*, 2003) or no quantitative yield losses, but significant reduction in fruit quality, and thus marketable yield reductions up to 40% (Spence *et al.*, 2006). In contrast to TMV and ToMV, the transmission of PepMV in hydroponic systems was not considered likely because the virus was not detected in nutrient solutions from PepMV-infested greenhouse tomato crops using serological assays (Spence *et al.*, 2006). In horticultural cultivation systems, not one but several pathogens may compete for nutrients and living space on the same plant. Little is known about the interactions of multiple pathogens co-infecting the same host, particularly in hydroponics (Malpica *et al.*, 2006). In the present work, the efficiency of PepMV transmission via the nutrient solution in a closed recirculating hydroponic system was studied by monitoring viral spread into the roots and different shoot organs of tomato plants during a growing period. This study also assessed possible interactive effects of PepMV and *P. aphanidermatum* by

quantifying the effects of single and mixed infections on pathogen infection rates and tomato growth and yield.

## Materials and methods

### PepMV inoculum production

The virus isolate PepMV-Sav (E397) was used in all experiments. It was initially recovered from tomato fruits showing yellow discoloration, spots and rings, obtained from a German supermarket and labelled to be imported from France. The isolate was recovered from these tomato fruits by maceration of crude fruit in ELISA sample buffer followed by mechanical inoculation, using 0.05% Celite as an abrasive, to tomato cv. Castle Rock (Peto Seed) or cv. Hildares (Hild) plants, on which it was propagated further.

### *Pythium aphanidermatum* inoculum production

*Pythium aphanidermatum* (BBA 70417) was obtained from the Federal Research Centre for Cultivated Plants (Julius Kühn Institut), Berlin, Germany. Culture and sample preparation of the isolate was performed according to Pannova *et al.* (2004).

### Climate-chamber experiments (E1 and E2)

In experiment E1 nine tomato seedlings of cv. Castle Rock were grown in 300 mL nutrient solution (pH 6.0; Steiner, 1961). Pots were spaced to avoid leaf contact between different plants. Two weeks after emergence, two leaves of four seedlings were mechanically inoculated with PepMV. Infection of inoculated plants was confirmed 1 week later by DAS-ELISA. The entire 300-mL volume of nutrient solution per pot was then collected weekly from PepMV-infected plants and supplied to the roots of four healthy test plants. One healthy control seedling was supplied with autoclaved nutrient solution only. Roots, stems, leaves, flowers or fruits were tested by DAS-ELISA for PepMV infection 1, 3, 5 and 7 weeks after application of nutrient solution commenced. The experiment was repeated once (E2), applying the same procedure, but this time inoculation was started when plants were 5 weeks old as compared to 3 weeks old in the first experiment. Details of these experiments are summarized in Table 1.

### Greenhouse experiments (E3–E5)

Greenhouse trials E3 and E4 were performed in two successive years, 2002 at Großbeeren and 2003 at Berlin. Tomato seedlings in rockwool cubes (100 × 100 × 70 mm<sup>3</sup>) were inoculated mechanically at the two-leaf stage (20 days after emergence) with PepMV-Sav. Infection was verified 1 week later by DAS-ELISA.

Roots of tomato plants were inoculated at the four-leaf stage using a suspension containing *P. aphanidermatum* mycelium. Infection of inoculum plants was verified

**Table 1** Overview of experiments performed: total duration (pre- and post-treatment), cultivars used, total number of plants (including controls), environmental conditions such as relative humidity (RH), temperature (T), mean daily radiation (PAR) and methods used for PepMV detection

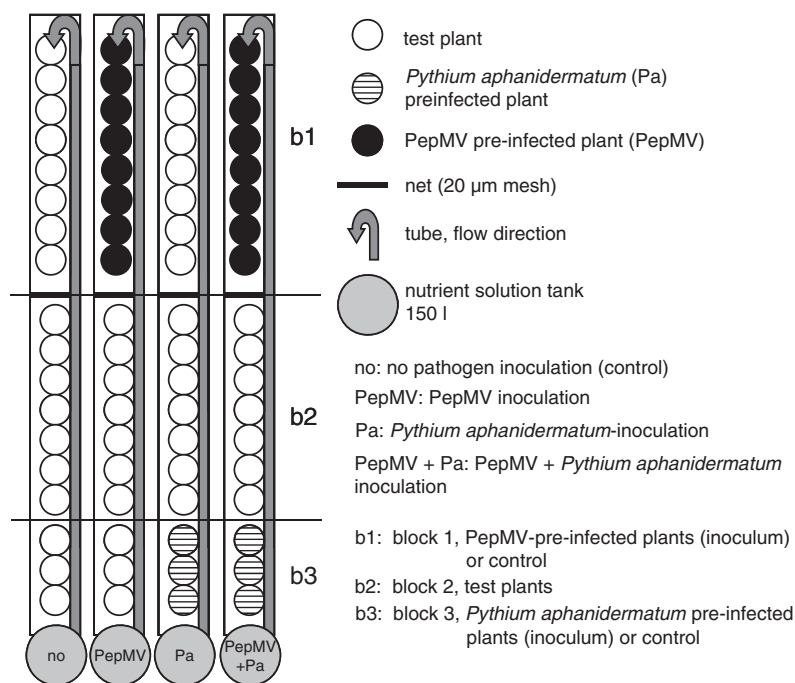
Experiment	Total duration (days)	Tomato cultivar	Plants (no.)	Location	RH (%)	T (°C)	PAR (MJ m <sup>-2</sup> day <sup>-1</sup> )	Detection methods
E1	21/49	Castle Rock	9 (1)	Growth chamber	73.0 ± 10	22.0 ± 0.7	3.3	ELISA, electron microscopy
E2	35/49	Castle Rock	9 (1)	Growth chamber	73.0 ± 10	22.0 ± 0.7	3.3	ELISA, electron microscopy
E3	50/102	Castle Rock	228 (54)	Greenhouse	83.7 ± 31	Mean 19.5 Min 5.0 Max 27.4	12.5 <sup>a</sup>	ELISA, indicator plants, electron microscopy, IC-RT-PCR
E4	50/107	Hildares	228 (54)	Greenhouse	65.1 ± 33	Mean 23.4 Min 19.8 Max 27.6	14.1 <sup>a</sup>	ELISA, indicator plants, electron microscopy, IC-RT-PCR
E5	40/65 20/56	Hildares <i>Nicotiana benthamiana</i>	8 (8)	Greenhouse	73.0 ± 24	Mean 22.0 Min 16.6 Max 33.5	6.8	ELISA, indicator plants, RT-PCR

<sup>a</sup>Outside the greenhouse.

7 days later (50 days after emergence) by randomized sampling of three root tips of about 10-mm length.

PepMV- and *P. aphanidermatum*-infected seedlings, together with healthy control and test plants of the same age, were transferred 50 days after emergence to gullies (8 m × 0.2 m × 0.1 m) according to Figure 1. Each of the four treatment rows was planted in three replicates and contained three blocks (b1–b3). Infected plants in b1 and

b3 served as the inoculum source for the healthy test plants (b2) in the same gully. In total, 12 gullies separated the four treatments and three blocks at a distance of at least 1 m. There was no plant contact between plants from different blocks. The distance within the row was 0.4 m, with a distance of 1 m between the last plant of one block and the first plant of the next block. A 20-µm mesh net separated both blocks to prevent root contact



**Figure 1** Schematic view of the treatment design in the greenhouse tomato experiments. Nutrient solution was supplied continuously with recirculation in four rows with different treatments. Seven test plants for each treatment were located in block b2. Root contact was possible for plants within one block, but not between plants of different blocks. *Pepino mosaic virus* (PepMV) and *Pythium aphanidermatum* (Pa) infection rates in block b2 (test plants) were analysed and compared to infection rates in b1 and b3 with *P. aphanidermatum*- and PepMV-pre-infected plants. The whole design was replicated three times in two experiments.

between infected inoculum plants in block b1 and test plants in block b2. Each gully was irrigated from a separate tank containing a pump continuously recirculating about 100 L of nutrient solution at a flow rate of about  $2 \text{ L min}^{-1}$  (De Kreij *et al.*, 1997; Fig. 1). Electrical conductivity was adjusted to  $2.5 \text{ dS m}^{-1}$  and the pH to 5.6, and was controlled manually three times a week. In 2002 the plants were grown from July to October; in 2003 the trial ran from June to September. Cultivation was performed following commercial practices. However, to prevent mechanical spread of the virus, plant handling was carried out using disposable rubber gloves that were changed after each plant in test block b1 and after each block in b2 and b3 for all four treatments. Three root fragments of about 10-mm length were randomly sampled every week from each plant of block b2 in both years. Additionally, all plants in block b1 were sampled in 2003. Plants were individually assessed weekly for *P. aphanidermatum* infection until infection of the plant root system was confirmed.

An additional greenhouse experiment (E5) was carried out from June to August 2008. Eight tomato plants spaced to avoid leaf contact between different plants were grown in 10-L containers filled with nutrient solution ( $2.5 \text{ dS m}^{-1}$ , pH 5.6, De Kreij *et al.*, 1997). Two tomato plants were supplied each week with 1 L nutrient solution removed from 10-L containers containing PepMV-infected plants. Thus, after 4 weeks all tomato plants had been inoculated by nutrient solution.

Details of all these experiments are summarized in Table 1.

### PepMV detection

In all experiments tomato plants were assessed visually for symptom development once a week. Plant tissues samples were tested by DAS-ELISA once a week (modified after Clark & Adams, 1977) using commercially available polyclonal antibodies (immunoglobulin IgG) according to the instructions provided (Prime Diagnostics, Plant Research International B.V.). Each ELISA test always included one positive and three negative controls. Samples were rated positive if the absorbance measured at 405 nm was greater than twice the level obtained from healthy controls (Córdoba-Sellés *et al.*, 2007). However, most positive readings were five to ten times greater. Seven samples with absorbance levels about twice those of healthy controls were investigated under the electron microscope to exclude putative false-positive ratings. Virus particles were detected in all these samples by electron microscopy. In addition, eight samples of randomly chosen plants that tested positive in ELISA were also inoculated to indicator plants *Nicotiana clevelandii* and *N. benthamiana*. Leaves of inoculated indicator plants tested positive for PepMV infection by ELISA in all cases. Eight randomly selected control plants tested by ELISA, electron microscopy and inoculation to indicator plants were negative for PepMV infection. In experiment E5, all tomato and *N. benthamiana* plants were also tested by

RT-PCR specific for the RNA-dependent RNA polymerase region (RdRP) of the PepMV genome, confirming the results from ELISA tests.

The nutrient solution from greenhouse experiments E3 and E4 was tested by ELISA and coat-protein-specific immunocapture reverse transcription PCR (IC-RT-PCR) using 80 mL nutrient solution from each treatment tank. The solution was concentrated by ultracentrifugation for 2 h at  $80\,695 \text{ g}$  and resuspended in  $100 \mu\text{L}$  Tris-HCl ( $0.01 \text{ M}$ , pH 7). For IC-RT-PCR, the same polyclonal antibodies were used for coating of tubes as were used in ELISA (1:250 v/v in ELISA coating buffer for 3 h at  $37^\circ\text{C}$ ). Homogenized plant material and concentrated nutrient solutions were incubated overnight at  $4^\circ\text{C}$  in coated tubes, washed three times with ELISA sample buffer followed by RT-PCR using the primer set described in Mumford & Metcalfe (2001).

In greenhouse experiment E5, 400 mL nutrient solution collected in equal portions from the eight containers with tomato plants were ultracentrifuged and processed as described before. After resuspension of the pellet, part of the obtained  $100 \mu\text{L}$  solution was used to inoculate three *N. benthamiana* indicator plants. Another part was used for RNA extraction carried out with the Invisorb Spin Virus RNA Mini Kit (Invitex) according to the protocol of the supplier. One microgram of RNA was used for cDNA synthesis with MMLV reverse transcriptase and  $0.5 \mu\text{g}$  oligodT primer following the manufacturer's recommendations (Promega). Three micrograms of the cDNA were used for PCR with  $0.5 \mu\text{M}$  primers PepMV-RdRp-Pep3-F and PepMV-RdRp-Pep4-R (Pagán *et al.*, 2006) and  $0.5 \text{ U}$  *Taq* polymerase under buffer conditions recommended by the supplier of the enzyme (peqlab). Amplification was carried out as described in Pagán *et al.* (2006) and PCR products cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's recommendations. Plasmid DNA from three clones was sequenced.

### Sampling PepMV, greenhouse experiments

In experiment E3 a leaf from the bottom of the plant (all plants) and five root fragments (third and seventh plants in b2, first plant in b3) were sampled weekly in b2 and b3 in the treatments with PepMV from the three replicated rows. In the same treatments, blocks and replications five root fragments, a young pinna (second leaf from the top) and three mature fruits were taken from all 10 plants weekly beginning 56 days after inoculation (d.a.i.). In experiment E4, the same sampling schedule was followed. However, all samples (roots, old and young leaves, as well as mature fruits) were sampled beginning 7 d.a.i.

In experiment E5 leaves were tested weekly by DAS-ELISA and RdRP-specific RT-PCR for PepMV infection. Leaves tested positive in ELISA also rated positive in RT-PCR. In addition the nutrient solution was tested for the presence of PepMV after ultracentrifugation by

inoculation to tomato and *N. benthamiana* test plants and by RdRP-specific RT-PCR (see above).

### Plant growth analysis (E3 and E4)

Fruits were harvested every week starting 49 d.a.i. in 2002 and 56 d.a.i. in 2003. Fresh and dry weights were determined (116 days after emergence). At the end of the experiment (77 and 91 d.a.i. in 2002 and 2003, respectively) fresh and dry weights of roots and shoots were determined. Dry weight was measured after drying subsamples in an oven at 80°C for 72 h.

### Statistical analyses (E3 and E4)

Data on PepMV infection and distribution were subjected to a non-parametric test. Means were compared by the Mann-Whitney *U*-test at significance level  $\alpha = 0.05$ . Other data measured were subjected to two-way analysis of variance. Means were compared by Fisher's *F*-test followed by Tukey's *t*-test at significance level  $\alpha = 0.05$ . Significant differences are represented by different letters or asterisks in the figures.

## Results

### Transmission of PepMV through the nutrient solution under climate-chamber conditions (E1 and E2)

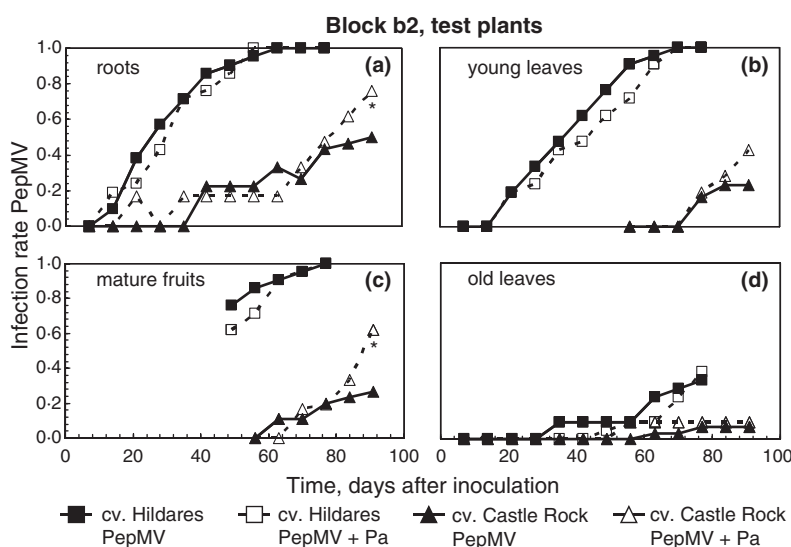
Transmission of PepMV to roots of healthy tomato cv. Castle Rock seedlings occurred through the nutrient solution within 7–35 days. No symptoms were observed at

any time. In E1, PepMV was detected in three out of four test plants 14 days and in all four plants 21 days after the first supply of drainage solution (absorbance  $1.16 \pm 0.67$ , negative control  $0.099 \pm 0.028$ ). In E2, PepMV was detected in one plant 7 days and in two more plants 28 and 35 days after the first inoculation (absorbance  $0.347 \pm 0.22$ , control  $0.099 \pm 0.031$ ). Weekly ELISA testing of the nutrient solutions from the four-inoculum producing plants, as well as that from the test plants, proved negative at all times. ELISA tests of leaves and green fruits remained negative for PepMV throughout the 77-day cultivation period. In contrast, ELISA confirmed infection of leaf and root material from the inoculum-producing plants throughout both experiments.

### Transmission and spread of PepMV through nutrient solution under greenhouse conditions (E3–E5)

Seedlings of tomato cvs Castle Rock and Hildares used as PepMV inoculum started to exhibit chlorosis and necrosis 7 days after mechanical inoculation. Plants showed some conspicuously crumpled and asymmetric leaves, but recovered 20 days later and remained symptomless during the remaining cultivation period.

In both experiments, roots of healthy test plants in b2 became infected with PepMV via the nutrient solution. Infection rate of roots was lower and virus spread was slower for Castle Rock in 2002 than for Hildares in 2003, i.e. first detection occurred 42 days vs. 14 d.a.i. (Fig. 2a). The infection rate for test plants at harvest time was 30% in 2002 and 100% in 2003. Fifty percent of Hildares plants were infected with PepMV 21 d.a.i., but it took 91 days for 50% of Castle Rock plants to become



**Figure 2** *Pepino mosaic virus* (PepMV) infection rates in (a) roots, (b) young leaves, (c) mature fruits and (d) old leaves of tomato test plants in block b2 (see Fig. 1) shown for two treatments, PepMV inoculation only (PepMV) and co-inoculation with PepMV and *Pythium aphanidermatum* (PepMV + Pa), for two cultivars, Castle Rock (2002) and Hildares (2003) grown in a closed recirculating hydroponic system. Data points represent mean values from  $n = 9$  plants up to 70 days after inoculation for cv. Castle Rock and from  $n = 21$  plants for all other results. Asterisks indicate significant differences between treatments within a year.

infected (mean absorbance in ELISA  $1.223 \pm 0.706$ , control  $0.09 \pm 0.012$ ). The infection of healthy Hildares plants occurred rapidly and followed no consistent patterns in the three replicated rows (mean absorbance in ELISA  $0.912 \pm 0.595$ , control  $0.130 \pm 0.027$ ).

PepMV was not detected in samples of nutrient solutions by coat-protein-specific IC-RT-PCR and ELISA in greenhouse experiments E3 and E4. However, it was detected in an additional greenhouse experiment (E5) by RdPR-specific RT-PCR after ultracentrifugation and RNA extraction from the resuspended pellet. Three independent clones of the 624-bp amplification product were found to be 99% identical to a French PepMV isolate (Accession No. AJ438767, Cotillon *et al.*, 2002). The partial coding sequence of the RdRP of 624 bp has been submitted to the EMBL Nucleotide Sequence Database (Accession No. FN386458). Partial sequence analysis of the coat-protein region after IC-RT-PCR from infected leaves, cloning and sequencing of three independent clones revealed that the deduced amino acid sequence (237 aa) of the coat-protein gene fragment was 99% identical to the sequences of the same isolate described by Cotillon *et al.* (2002) and also to other isolates from France, Spain and the UK (e.g. isolate UK2, Accession No. AF340024), revealing a close relationship of this part of the genome of isolate PepMV-Sav to the European genotype of PepMV. The 847-bp nucleotide sequence has been submitted to the EMBL Nucleotide Sequence Database (Accession No. AM930243).

Inoculation of *N. clevelandii* and *N. benthamiana* indicator plants with samples of the nutrient solution from all greenhouse experiments before and after ultracentrifugation resulted in typical symptoms, such as distorted leaves. Here, PepMV was detected by ELISA and RT-PCR, indicating the presence of infectious PepMV particles in quantities below the detection limits of the ELISA and IC-RT-PCR tests in the nutrient solution.

In experiment E5 leaves of one tomato cv. Hildares plant tested positive in ELISA 14 d.a.i., which was similar to the time of first detection in experiment E4. After a period of a further 21 days, six of eight tomato (75%) and seven of eight *N. benthamiana* (87%) plants tested positive (absorbance  $0.246 \pm 0.10$ , control  $0.102 \pm 0.01$ ). RT-PCR testing of leaves confirmed infection by PepMV for all plants. Direct inoculation of *N. benthamiana* plants with nutrient solution concentrated by ultracentrifugation revealed typical symptoms. RT-PCR of leaves confirmed PepMV infection. Leaf material of infected plants was back-inoculated to three 20-day-old tomato cv. Hildares seedlings. After 7 days plants did not show symptoms, but tested positive for PepMV by ELISA and RT-PCR.

#### Distribution of PepMV within the plant (E3 and E4)

Roots became infected first, as soon as 14 d.a.i. (Fig. 2a). As little as 7 days after root infection was detected, serological tests confirmed PepMV infection in young leaves (second leaf from top, Fig. 2b). Infection of older leaves

(fifth from the bottom) was detected 35 d.a.i. in less than 33% (Hildares) or 7% (Castle Rock) of plants (Fig. 2d). In Hildares, 76% of the first mature fruits (harvested 49 d.a.i.) were infected with PepMV (Fig. 2c) and by 77 d.a.i., 100% of mature fruits sampled tested positive for PepMV (Fig. 2c). By contrast, in Castle Rock at harvest start, 56 d.a.i., no mature fruits tested positive, whereas at harvest end, 91 d.a.i., 27% of mature fruits were infected with PepMV. The time course of infection of mature fruits and young leaves in the greenhouse experiments followed closely the time course of root infection, delayed by about 7–14 days. By the end of experiment E4, 75% of all young leaves in the top part of the plant, 25% of leaves in the middle and 17% of old leaves in the lower part were infected of the six plants tested.

#### Spread of *Pythium aphanidermatum* (E3 and E4)

Infection by *P. aphanidermatum* within the healthy test plants in block b2 was first confirmed in roots 28 days (Castle Rock) or 7 days (Hildares) after inoculation. All plants were infected 56 and 21 d.a.i. (Castle Rock and Hildares respectively).

#### Interactions between PepMV and *Pythium aphanidermatum* (E3 and E4)

Infection progress with *P. aphanidermatum* appeared slower in Hildares when plants were pre-infected with PepMV (b1) compared with plants not pre-infected (b2), but 21 d.a.i. 100% of plants were infected with *P. aphanidermatum* in both treatments.

Pre-infected (with *P. aphanidermatum*) plants of Hildares, but not Castle Rock, in b3 succumbed to infection with PepMV in roots, leaves and fruits significantly later than non-pre-infected plants (Fig. 3). Twenty-eight days after treatment start, roots of all pre-infected plants were still PepMV-negative, while 67% of the non-pre-infected plants were positive (Fig. 3a). The largest difference for young leaves was 56 days after treatment start, when pre-infected plants showed 22% PepMV-positive plants and non-pre-infected plants showed 78% PepMV-positive plants (Fig. 3b); similar results were found for the infection rates in mature fruits (22 and 67%, respectively, Fig. 3c). However, 70 d.a.i. roots, fruits and young leaves of all plants were 100% infected both in the pre-infected and non-pre-infected plants. In the old leaves, however, an infection rate of only 10% was seen in the pre-infected treatment at the end of the experiment, as compared to 55% in the non-pre-infected plants.

#### Growth and yield (E3 and E4)

Plant growth analysis in Castle Rock and Hildares test plants (b2), revealed reduced fresh matter, dry matter and yield for all treatments compared with untreated controls (Fig. 4). PepMV infection resulted in a significant reduction in total yield of Castle Rock (23.3%), but not in fruit

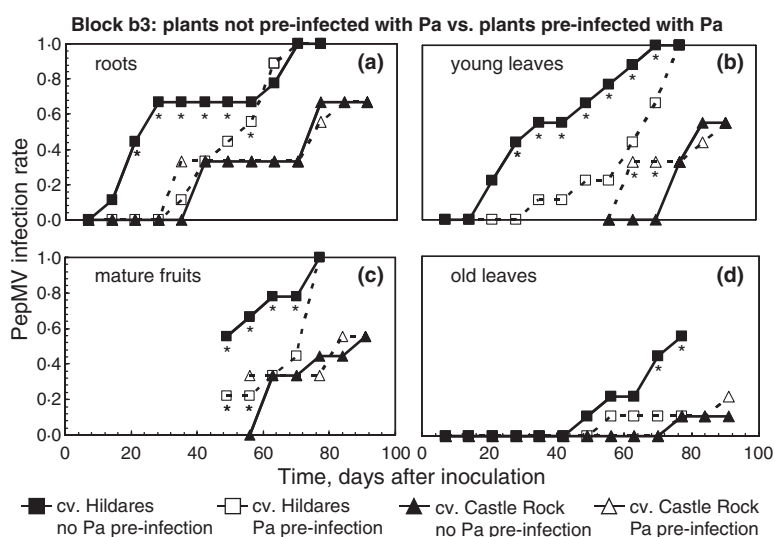


Figure 3 *Pepino mosaic virus* (PepMV) infection rates in tomato plants pre-infected with *Pythium aphanidermatum* (Pa) versus plants without pre-infection in block b3 (see Fig. 1) in (a) roots, (b) young leaves, (c) mature fruits and (d) old leaves shown for two cultivars, Castle Rock (2002) and Hildares (2003) grown in a closed recirculating hydroponic system. Data points represent mean values from  $n = 9$  plants in block b3 for both cultivars. Asterisks indicate significant differences between treatments within a year.

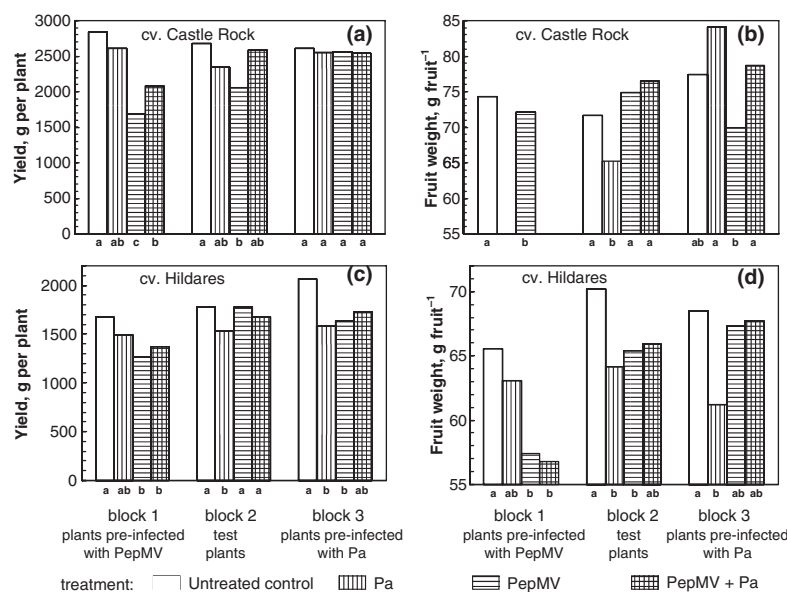


Figure 4 Yield characteristics of tomato plants in three blocks (see Fig. 1) with four different treatments: untreated control, inoculated with *Pepino mosaic virus* (PepMV), inoculated with *Pythium aphanidermatum* (Pa), or co-inoculated with both pathogens (PepMV + Pa). Block b1, plants pre-infected with PepMV; block b2, healthy test plants; block b3, plants pre-infected with *P. aphanidermatum*. (a, c) Total yield in g per plant; (b, d) single-fruit weight; (a, b) cv. Castle Rock; (c, d) cv. Hildares. Different letters indicate significant differences within an experiment at  $P = 0.05$ .

weight (Fig. 4a,b). Fruit weight of Hildares was reduced (by 7%) compared with the untreated control, but total yield was not (Fig. 4c,d). Yield reduction caused by *P. aphanidermatum* was significant only for Hildares, but fruit weight was reduced by about 9% in both cultivars. However, co-inoculation with both pathogens did not result in a more pronounced yield reduction. On the

contrary, the yield reduction was only 3.3 and 5.9% for Castle Rock and Hildares, respectively (Fig. 4a,c).

In the PepMV treatment, pre-infection with PepMV in block b1 resulted in the greatest (and significant) yield reduction compared with non-PepMV-pre-infected test plants. Yield reduction was 40.4% for Castle Rock and 24.3% for Hildares. Pre-infection with

*P. aphanidermatum* in block b3 significantly reduced the yield of Hildares (11%), but not of Castle Rock. Neither yield nor fruit weight of Castle Rock was reduced in the treatment with both pathogens. Total yield of Hildares was lower than that of Castle Rock. However, *P. aphanidermatum* pre-inoculation in block 3 resulted in a significant reduction of total yield and single-fruit weight of Hildares (Fig. 4c,d), whilst treatment with both pathogens affected only single-fruit weight.

## Discussion

In each of two climate-chamber and three greenhouse experiments PepMV was readily transmitted through nutrient solution and was able to infect a high percentage of tomato plants of two cultivars efficiently within 1–6 weeks. This indicates that in addition to other known modes of transmission, e.g. spread by tools contaminated with infected plant sap, the spread of PepMV in hydroponic systems via the nutrient solution and roots of plants can have an important epidemiological significance.

Experiments in controlled-environment chambers revealed that infectious PepMV particles are released into the nutrient solutions from infected tomato roots and can infect test plants without direct root contact. Roots of seven out of eight test plants were infected within 1–5 weeks after incubating them in nutrient solution obtained from PepMV-infected tomato plants. Roots of seedlings of cv. Castle Rock, 3-weeks-old at the time when inoculation started, seemed to be more efficiently infected through the nutrient solution than roots of 5-week-old seedlings.

Similarly, PepMV was efficiently transmitted from infected roots to test plants through the nutrient solution in a typical hydroponic greenhouse tomato production set-up within 2–6 weeks. In this hydroponic system, contact between roots or root fragments was prevented by filtering the nutrient solution through a 20- $\mu$ m mesh and by physical separation of the test plants from the inoculum plants. Consequently, initial infection of the test plants could only occur through the drainage nutrient solution. Planting distances of 0.4 m between single tomato plants within the test plant block mimicked commercial hydroponic tomato production, allowing root and leaf contact between adjacent plants. Thus, the experimental set-up could not distinguish unambiguously between subsequent virus transmission through the nutrient solution and transmission through leaf or root contact between adjacent plants within test blocks following initial PepMV infection. However, in greenhouse experiment E5 the virus must have been transmitted exclusively by the nutrient solution because leaf contact between plants was prevented.

Although efficient transmission of plant viruses through nutrient solution in hydroponic systems has been described for plant viruses from different taxa and for different host-plant species, including tomato [e.g. *Tomato bushy stunt tomosvirus*, *Cymbidium ringspot tomosvi-*

*rus*, *Odontoglossum ringspot tobamovirus*, *Cymbidium mosaic potexvirus* and ToMV (Pares *et al.*, 1992)], the epidemiological relevance of direct transmission through the nutrient solution and transmission by root contact or root-grafting for virus transmission has rarely been studied. For TMV transmission between tomato plants in a hydroponic system occurred exclusively by root-grafting, not by direct transmission through the nutrient solution (Park *et al.*, 1999). The results of the present climate-chamber experiments and the time pattern of initial virus detection in neighbouring and distantly positioned plants within rows in the greenhouse experiments suggested that transmission of viral infectious units through the nutrient solution contributed to PepMV spread.

Although the presence of PepMV viral particles in the nutrient solution could not be demonstrated directly using ELISA or IC-RT-PCR in greenhouse experiments E3–E5, PepMV was detected directly in the nutrient solution after concentration by ultracentrifugation followed by RT-PCR in greenhouse experiment E5. Also, inoculation of nutrient solution directly to indicator plants led to successful infection with PepMV, indicating the nutrient solution contained infectious PepMV particles or RNA below the ELISA and IC-RT-PCR detection limit. In greenhouse trials performed by Spence *et al.* (2006) all runoff water from rockwool slabs also tested negative for PepMV using a lateral flow diagnostic test kit, despite test plants being severely infected, and it was concluded that risks from recycled irrigation water may be limited. However, the results here indicate that even with very low viral titres, efficient transmission of PepMV to healthy tomato plants through the nutrient solution is possible. In contrast, Park *et al.* (1999) reported that TMV-infected tomato plants release TMV particles into the nutrient solution, producing high virus titres in a hydroponic system that can be detected by ELISA even before the TMV concentration reaches the disease threshold level. The transmission of PepMV, even at very low concentrations, indicates that the virus is highly contagious and has a lower disease threshold than TMV in hydroponic systems (Hanssen *et al.*, 2008b).

Yield loss, measured either as yield per plant or fruit weight in the PepMV treatments (50–100% plants infected at harvest time) amounted to 0.4–40.4%, depending on treatment and experimental conditions. The reduction in yield for cv. Hildares was more pronounced in pre-infected plants and less pronounced in test plants succumbing to PepMV infection during the trial than for cv. Castle Rock. PepMV-pre-infected Castle Rock plants showed the highest yield reduction. These plants were mechanically inoculated early in the second leaf stage (20 days after emergence). This indicates that young seedlings are more sensitive to PepMV infections, resulting in a higher yield decrease, as has been described for many other plant viruses. In contrast to the present observations, Spence *et al.* (2006) reported that in Dutch tomato production greenhouses it is common practice to inoculate plants with PepMV at an early stage, as late infections are believed to be much more detrimental to



fruit quality. However, the trials here indicate that yield losses can be more pronounced when plants are infected at an early stage. Yield losses of less than 5% in the Netherlands (van der Vlugt *et al.*, 2002) and 15–90% in Spain (Jordá *et al.*, 2001; Soler-Aleixandre *et al.*, 2005) have been reported. The high variability of these reports suggests that the impact of PepMV infection on tomato yield depends on interacting factors, including climate conditions, tomato genotype and virus isolate. This might also account for differences between the present findings and those of Spence *et al.* (2006) and Soler-Aleixandre *et al.* (2005). In contrast to the present results, Spence *et al.* (2006) reported that bulk yields in a similar trial of two cultivars were not reduced, but fruit quality was reduced significantly. Reports on yield decreases and fruit quality reductions caused by PepMV are conflicting, most reports being based on farmers' observations rather than experimental trials. Fruit discoloration, reduced size, or other fruit quality traits which would result in sales price reduction did not occur. The lack of symptoms in both greenhouse experiments and throughout the entire trial period contrasts with some studies (Spence *et al.*, 2006), but is similar to reports from Soler-Aleixandre *et al.* (2005) and from observations in many greenhouses in Belgium and the Netherlands (I. M. Hanssen, personal communication).

One question resulting from the present findings is whether the variation in disease severity between both greenhouse experiments was primarily caused by environmental or genetic factors. Different PepMV genotypes have been shown to cause similar symptoms in commercial tomato crops, except when present in mixed infections (Hanssen *et al.*, 2008a). Additionally, no obvious differences in symptom expression, virus titre, disease severity or resistance were found in 23 tomato accessions from a tomato germplasm collection (Ling & Scott, 2007). No significant differences were found in symptom expression and ELISA titres between seven tomato cultivars infected with the same PepMV isolate used in the present study and with another isolate (unpublished data). Thus, environmental factors probably have a larger impact on the variation in disease severity observed in the greenhouse experiments between different years than genetic factors. Environmental factors, such as light and temperature, are thought to play a crucial role in symptom development and disease severity. Symptom severity was reported to decrease with an increase of temperature (Jordá *et al.*, 2001; Pagán *et al.*, 2006). However, in the present experiments the most severe PepMV infection and yield reduction occurred in 2003, the year with the higher mean temperature (23.4°C, compared with 19.5°C in 2002) and the lower temperature range (7.5°C, compared with 22.5°C in 2002). The data reported here show that yield reduction in tomato production can be expected upon PepMV infection of tomatoes. However, the impact on yield is difficult to predict because of the influence of factors described above.

Although multiplex pathogen–host interactions are very common in commercial production systems, most

research on pathogen epidemiology has dealt with specific single pathogen–host interactions. For hydroponic tomato production systems a few reports are available on interactions between common oomycete and fungal and bacterial pathogens, e.g. *Pythium* spp. and *Pseudomonas* spp. (Gravel *et al.*, 2005), and between common viral pathogens, e.g. *Potato virus X* and TMV (Balogun, 2003). According to Spence *et al.* (2006) a report from the Netherlands has indicated that PepMV and *Verticillium* sp. may act synergistically, seriously reducing the yields of co-infected plants. Because of the lack of reports on the interaction of the important pathogens *P. aphanidermatum* and PepMV, the present work studied the mutual influence of these two pathogens on their spread and on tomato yield. Single treatment with PepMV and double treatment with PepMV and *P. aphanidermatum* did not result in differences in PepMV spread, infection rate, plant growth and yield in cvs Castle Rock and Hildares. However, pre-infection of Hildares with *P. aphanidermatum* resulted in a significant delay of root infection with PepMV (49 days), although growth and yield were not affected. The root necrosis caused by *P. aphanidermatum* could perhaps induce resistance mechanisms affecting virus multiplication and spread (Van Loon, 1997) and/or biochemical and structural changes in root architecture, reducing the efficiency of PepMV uptake of roots through the nutrient solution.

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