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Pepino mosaic virus isolates and differential symptomatology in tomato

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Based on a survey conducted in commercial tomato production in Belgium in 2006, four *Pepino mosaic virus* (PepMV) isolates that differed in symptom expression in the crop of origin were selected for greenhouse trials. The selected isolates were inoculated onto tomato plants grown in four separate plastic tunnels. PepMV symptom development was assessed regularly and extensive sampling followed by ELISA analyses, genotyping and sequencing was performed to study viral presence and variation in PepMV sequences throughout the trial period. Two isolates (EU-mild and CH2-mild) that were selected based on mild symptom expression in the crop of origin caused only mild symptoms in the trial, while two other isolates (CH2-aggressive and EU + CH2) that were selected for severe symptom display, caused considerably more severe symptoms. Sequence homology between CH2-mild and CH2-aggressive was as high as 99·4%. Results of this study show that differential symptom expression can, at least partially, be attributed to the PepMV isolate, which may be related to minor differences at the nucleotide level between isolates.

Keywords: greenhouse trial, pathogenicity, PepMV, *Potexvirus*, *Solanum lycopersicum*

Introduction

In recent years, tomato (*Solanum lycopersicum*) greenhouse crops have been increasingly affected by the highly contagious potexvirus *Pepino mosaic virus* (PepMV), which was first described in 1980 on pepino (*Solanum muricatum*) in Peru (Jones *et al.*, 1980). Infection of tomato was not reported until 1999, when the virus was first detected in tomato greenhouse crops in the Netherlands (Van der Vlugt *et al.*, 2000). Since then, rapid spread of the virus occurred throughout tomato production worldwide, with official reports of PepMV incidence from Spain, France, Italy, the UK, Poland, Belgium, the USA and Canada (Soler *et al.*, 2000; French *et al.*, 2001; Jorda *et al.*, 2001; Mumford & Metcalfe, 2001; Roggero *et al.*, 2001; Cotillon *et al.*, 2002; Pospieszny & Borodynko, 2006; Hanssen *et al.*, 2008).

The PepMV viral RNA genome is approximately 6400 nucleotides long and, similar to other potexviruses, contains five open reading frames (ORFs), including a replicase gene, a triple gene block (TGB) and a coat protein gene (CP). Sequence data revealed a high genome identity (approximately 99%) between isolates from different European countries (Aguilar *et al.*, 2002; Cotillon *et al.*, 2002; Mumford & Metcalfe, 2001; Verhoeven *et al.*, 2003). Since these European isolates showed only 96% sequence homology to a Peruvian PepMV isolate (LP 2001), they are considered as a distinct genotype and further referred to as the EU tomato genotype (López *et al.*, 2005). Two other PepMV isolates, originating from diseased tomato plants in the USA (US1 and US2; Maroon-Lango *et al.*, 2005), displayed between 79% and 82% homology to the EU tomato genotype. A fifth genotype, the so-called CH2 genotype, which showed more sequence homology with the US2 genotype (91%) than with the EU tomato genotype (about 80%), was found on contaminated tomato seeds originating from Chile (Ling, 2007).

A phylogenetic study in Spanish tomato production revealed the presence of the Peruvian and US2 genotypes in addition to the EU tomato genotype of PepMV (Pagan *et al.*, 2006). Recently, the CH2 genotype has spread

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throughout Europe and was reported to occur in Belgium (Hanssen *et al.*, 2008) and Poland (Hasiów *et al.*, 2008), but has also been identified in France, the Netherlands and Morocco (Hanssen *et al.*, unpublished data). Genetic characterization of PepMV isolates from Belgian greenhouse tomatoes demonstrated dominance of the CH2 genotype over the EU tomato genotype and the frequent occurrence of recombinants between both genotypes in mixed infections (Hanssen *et al.*, 2008).

Initially, symptoms such as nettle-heads, dwarfing, leaf distortions, leaf mosaics, yellow rectangular leaf spots and marbling or flaming of fruit were associated with PepMV infection in tomato. As leaf or head symptoms were usually mild and persisted only for a short period after infection, fruit discoloration was generally considered the most devastating consequence of PepMV infection (Soler *et al.*, 2000; Roggero *et al.*, 2001; Spence *et al.*, 2006). Nevertheless, since the emergence of new PepMV genotypes, symptom severity seems to be increasing as not only the common leaf and head symptoms are becoming more persistent and severe, but also new symptoms (e.g. leaf scorching or premature leaf senescence, open fruit and scars on the fruit surface) are observed (Spence *et al.*, 2006; Hanssen *et al.*, 2008). So far, no correlation has been observed between different PepMV genotypes and the severity of symptom expression in infected tomato plants (Pagan *et al.*, 2006; Hanssen *et al.*, 2008). However, co-infection with both genotypes resulted in enhanced PepMV symptoms (Hanssen *et al.*, 2008). Subsequently, a detailed survey of symptom display in Belgian greenhouse tomatoes infected with PepMV in 2006 gave rise to the hypothesis that severity and nature of symptoms induced in tomato plants differ between isolates, and even within the same genotype. From this survey, four PepMV-isolates, obtained from these naturally infected commercial tomato crops were selected for further study. The study reported here compared PepMV symptom expression caused by these four different PepMV isolates in greenhouse trials. In addition, complete genome sequences were determined and sequence evolution during infection was assessed.

Materials and methods

Experimental design

From May to October 2007, a greenhouse trial was conducted in five separate plastic tunnels to assess PepMV symptom expression upon inoculation with different PepMV isolates. Tomato seeds (cv. Tricia, De Ruiter Seeds) were disinfected with sodium hypochlorite (1° active chlorine for 30 min) and rinsed thoroughly with tap water. Subsequently, the seeds were germinated on rockwool trays in a lettuce greenhouse facility, isolated from tomato production facilities to prevent PepMV infection. Five weeks after sowing, 100 tomato plants were transferred to each of the tunnels. One month after planting, the tomato plants in four separate tunnels were individually inoculated with four different PepMV strains, while plants in a fifth tunnel were mock-inoculated with phosphate buffer.

Based on a survey conducted in 2006 in commercial tomato production greenhouse facilities in Belgium, four PepMV isolates (1806, 1906, 0506 and PCH 06/104) obtained from different greenhouses with distinct PepMV symptom expression in tomato were selected (Hanssen *et al.*, 2008). Here, a PepMV isolate is defined as the viral inoculum derived from PepMV-infected plants from one specific tomato production site. Inoculation was performed by rubbing one lower leaf per plant with an extract of infected tomato leaf material, prepared by grinding 30 g of leaf material in 60 mL phosphate buffer (pH 7·4). Viral concentration in the PepMV inoculum of the four isolates was standardized by using infected leaf material with a similar viral titre, as determined by ELISA. This method was assured to result in 100% disease incidence.

Each tunnel with 100 plants was divided into 10 sampling blocks, each containing 10 adjacent plants. Until the fourth week post-inoculation (WPI), a weekly sample consisting of a single leaf from the head of each of the 10 plants in the sampling block was taken from all 10 sampling blocks per tunnel. After 4 weeks, sampling was performed once every 2 weeks. An overview of the sampling schedule is given in Table 1.

Genetic characterization of PepMV isolates

The genotypes of PepMV isolates were determined using a previously described RT-PCR-RFLP method (Hanssen *et al.*, 2008). Whole genome sequences of the three isolates containing a single genotype (EU-mild, CH2-mild and CH2-aggressive) were determined by amplifying, cloning and sequencing seven partially overlapping regions in the PepMV genome (Table 2). Amplified products were directly cloned into the pCR4-TOPO vector (Invitrogen) and sequenced using the vector-specific primers M13-F and M13-R (Macrogen Inc.). Alignment of the full genome sequences was performed using the CLUSTAL X algorithm (Thompson *et al.*, 1997). In addition, for the EU + CH2 isolate, a 625-bp fragment of the genome, referred to as RdRp (Pagan *et al.*, 2006; Hanssen *et al.*, 2008), obtained using primers Pep3 and Pep4 (Table 2; Mumford & Metcalfe, 2001), was cloned and a total of 10 clones was sequenced.

Evaluation of PepMV symptoms

During the trial period, at 10 time points (0, 1, 2, 4, 5, 6, 8, 9, 10 and 14 WPI) 30 individual plants from each treatment were examined for PepMV symptoms. In addition, at three points in time (3, 7 and 12 WPI) all 100 plants were evaluated. In each case, the head of the plants, the foliage and stem, and the fruit were examined. Symptoms were rated using a scale from one (not present) to four (severe symptom display) (Table 3). In addition, at 9, 13 and 15 WPI all tomatoes in the mature clusters from each treatment were examined to determine the percentage of marbled and flamed fruits.

Table 1 Schedule of sampling and *Pepino mosaic virus* analyses on tomato

		PepMV analyses		
WPI[a]	Sampling date[b] (2007)	ELISA	Genotyping[c]	Sequencing[d]
1	20 June	10 samples per tunnel	/[e]	1 sample per tunnel, 7–10 clones
2	29 June	10 samples per tunnel	/	/
3	05 July	10 samples per tunnel	/	1 sample per tunnel, 7–10 clones
4	13 July	10 samples per tunnel	/	/
6	27 July	10 samples per tunnel	3 samples per tunnel (sampling blocks 1, 5 and 10)	/
8	10 Aug.	10 samples per tunnel	/	/
10	24 Aug.	10 samples per tunnel	/	/
12	07 Sep.	10 samples per tunnel	/	1 sample per tunnel, 7–10 clones
14	20 Sep.	10 samples per tunnel	/	/
16	01 Oct.	10 samples per tunnel	3 samples per tunnel (sampling blocks 1, 5 and 10)	1 sample per tunnel, 7–10 clones

[a]Weeks post-inoculation.
[b]A sample consisted of a mixture of 10 young leaves (one from each plant in the sampling block) from the heads of the plants. As the heads of the plants were pruned in the sixth WPI, leaves were taken from young shoots in the highest plant parts after 6 WPI.
[c]By RT-PCR-RFLP.
[d]RdRp fragment, after cloning (analysis not performed for EU + CH2 isolate 0506).
[e]Analysis not performed.

Table 2 Primer sets used for amplification and sequence determination of the *Pepino mosaic virus* genome

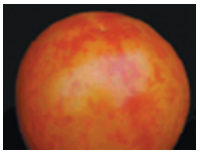
Primer[a]	Target region	5′ Position[b]	Sequence (5′–3′)	T_{ann}[c]	Amplicon size
Apa15	Replicase gene	36	CTAACACAACATAACCACG	57°C	1172
Rep1-R1		1190	GTTGCATGGGTGCAACCA		
Rep2-F	Replicase gene	1075	GAATTGTATGACCCTGATG	54°C	1316
Rep2-R		2371	GGTTGAATCATTGCTTTCTC		
Rep3-F2	Replicase gene	2166	TCAAAATGCAACATGAAGAC	54°C	1105
Rep3-R		3252	GTTGATGTTGGAAAAGTTG		
Rep4-F1	Replicase gene	2951	ACACCATATCTCAAAGC	51°C	1160
Rep4-R		4094	CCTTTAACCTGTTTTGG		
Pep3-F[d]	Replicase gene	3893	ATGAGGTTGTCTGGTGAA	53°C	625
Pep4-R[d]		4500	AATTCCGTGCACAACTAT		
Apa23-F	Triple gene block	4411	GTTTTCCCCAGTTTGAAATGG	54°C	1147
Apa25-R		5537	CCAAGGGGAGAAGTTGATTGC		
Ker1	Coat protein gene	5379	CACCAATAAATTTAGTTTTAGC	56°C	996
FL-R		6359	AGAAAACCCACTCTGA		

[a]F is sense primer, R is antisense primer.
[b]Reference sequence CH2, GenBank Accession number DQ000985.
[c]Annealing temperature.
[d]Mumford & Metcalfe, 2001.

Determination of PepMV presence and infection level

All plant samples were analysed for PepMV presence using a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using a commercial antiserum (Agdia Inc.), according to the supplier's instructions. Optical density (OD) was measured at 405 nm and samples were rated positive if the OD exceeded the mean value of two negative control wells by three times. Assay reproducibility was confirmed by including a duplicate sample of a standardized positive control of known viral concentration in each ELISA analysis. A mean OD value of 3.76 with a 95% confidence interval of 3.72–3.80 was obtained for the positive controls in each of the ELISA analyses, demonstrating the reproducibility of the analysis. The linear range of OD values was determined using a dilution series of the positive control of known viral concentration and ranged from 0·6 to 3·8. Since nearly all OD values for the samples from this trial fell within this range, the sample OD was considered as an indication for the PepMV viral titre. OD values per tunnel and per sampling point were determined by calculating the mean of the 10 OD values obtained from each sampling block.

Table 3 *Pepino mosaic virus* symptom rating scale for tomato

Plant part	Symptom type	Score	Description	
Head[a]	Nettle-head	1	Absent	
		2	Leaves are somewhat pointed and upright with a slightly reduced surface	
		3	Leaves are pointed, upright or curled, with a reduced surface	
		4	Leaves resemble nettle leaves, with a serrated leaf margin and a reduced surface	
	Leaf bubbling	1	Absent	
		2	One bubbled leaf[b]	
		3	Two to four bubbled leaves[b]	
		4	All leaves are bubbled[b]	
Foliage[c]	Premature leaf senescence	1	Absent	
		2	Scorching-leaflet margins	
		3	Scorching-entire leaflets of min. one leaf[b]	
		4	Scorching-more than one leaf[b]	
Fruit	Marbling	1	Absent	
		2	One marbled fruit[b]	
		3	Two marbled fruits	
		4	More than two marbled fruits[b]	
	Flaming	1	Absent	
		2	One flamed fruit[b]	
		3	Two flamed fruits[b]	
		4	More than two flamed fruits[b]	
	Open fruit	1	Absent	
		2	One open fruit[b]	
		3	Two open fruits[b]	
		4	More than two open fruits[b]	
	Necrcsis of the sepals	1	Absent	
		2	One fruit with sepal necrosis[b]	
		3	Two fruits with sepal necrosis[b]	
		4	More than two fruits with sepal necrosis[b]	

[a]Upper youngest leaves (plant top).
[b]Per plant.
[c]Lower leaves.

Viral genome variation

For the three single isolates used in this study, viral genome variation was studied by cloning and sequencing the 625-bp RdRp fragment (Mumford & Metcalfe, 2001; Pagan *et al.*, 2006; Hanssen *et al.*, 2008). From each treatment, four samples obtained at different time points (1, 3, 12 and 16 WPI), were used. Amplified products were

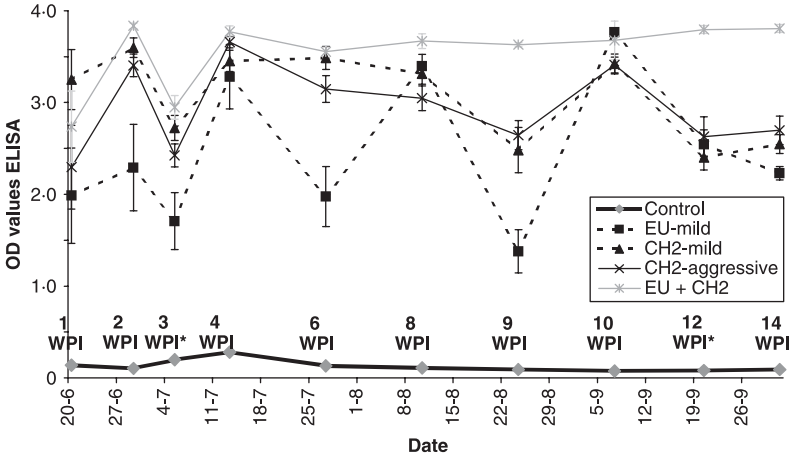


Figure 1 Viral accumulation in different *Pepino mosaic virus* inoculation treatments on tomato, displayed as mean optical density (OD) values obtained from 10 samples per tunnel (one sample from each sampling block) and per sampling point (indicated using the number of weeks post-inoculation (WPI) and the actual date on the horizontal axis), throughout the trial period. Standard errors bars are indicated for each measurement. Each sample consisted of a mixture of 10 young leaves (one from each plant in the sampling block) from the heads of the plants. As the heads of the plants were pruned in the sixth WPI, leaves were taken from young shoots in the highest plant parts after 6 WPI.

directly cloned and sequenced (7–10 clones per sample) as described above. Multiple sequence alignments were performed using CLUSTAL X (Thompson *et al.*, 1997) and neighbour-joining trees were constructed and displayed using TREEVIEW v. 1.6.6 (Page, 1996).

Results

Differential PepMV infection levels

A greenhouse trial was conducted in five separate plastic tunnels that each contained 100 cluster tomato plants, to assess PepMV symptom expression upon inoculation with four different PepMV isolates that were obtained from commercial greenhouse tomato crops with distinct PepMV symptoms (Hanssen *et al.*, 2008). PepMV isolate 1806 (EU-mild) belonged to the EU tomato genotype and did not cause typical PepMV symptoms in the tomato crop of origin. Isolate 1906 (CH2-mild) belonged to the CH2 genotype and caused mild symptoms. Isolate PCH 06/104 (CH2-aggressive) also belonged to the CH2 genotype, but caused severe PepMV symptoms in the crop of origin. Finally, isolate 0506 (EU + CH2), containing both the EU tomato and the CH2 genotype, caused severe symptoms. To verify that only the inoculated genotype was present in the PepMV-infected plants, genotyping analyses were performed using a previously described RT-PCR-RFLP genotyping method (Hanssen *et al.*, 2008) on samples harvested at six and 16 WPI (Table 1), confirming that no cross-contamination occurred between treatments throughout the trial period (data not shown).

ELISA analyses on samples taken 1 day before inoculation confirmed that the plants were not infected with PepMV prior to inoculation. One week after inoculation (1 WPI), however, the majority of the samples tested positive for PepMV. For the EU-mild and CH2-aggressive treatments, three out of 10 sampling blocks tested negative, while in the CH2-mild and the EU + CH2 treatments, only one sampling block tested negative. At 2 WPI, all sampling blocks tested positive, except for the same three sampling blocks of the EU-mild treatment. Of these three blocks, one tested positive from 3 WPI onwards, one from 4 WPI onwards and one from 6 WPI onwards. These results suggest that the EU-mild isolate colonized the tomato crop more slowly than the other isolates (Fig. 1). The viral titres observed in the different samples appeared to be different for the various treatments (Fig. 1). The OD values obtained for the EU-mild isolate were considerably lower at most of the sampling points, although plants in this tunnel displayed the largest variation in viral titres between subsequent sampling points (Fig. 1). At the end of the trial period, all samples contained a similar viral load (OD ~ 2·5), except for the CH2 + EU-inoculated plants (OD ~ 3·8), which contained a viral load at least 10 times higher. Samples from the mock-inoculated control tunnel remained negative in the ELISA assay throughout the trial period.

Differential symptom expression upon PepMV inoculation

Typical PepMV symptoms were monitored at regular intervals during the trial period (Fig. 2; Table 3). The same 30 plants were monitored weekly per treatment, except for 3, 7 and 12 WPI, when all 100 plants were monitored.

Typical nettle-head symptoms were seen from 2 WPI onwards (Fig. 2a). From 2 to 5 WPI, the most severe display of nettle-heads was found in plants inoculated with the CH2-aggressive or the EU + CH2 isolate, with the most nettle-head scores at 6 WPI. In contrast, inoculation with CH2-mild resulted in considerably less severe symptoms. No nettle-head-like symptoms were seen in the EU-mild-inoculated plants or in the mock-inoculated control (Fig. 2a). A similar pattern of symptom expression was recorded for bubbling of the leaf surface in the head of the plants (Fig. 2b). At 6 WPI, scoring of nettle-head symptoms and bubbling of the leaf surface was terminated because the heads of the plants were pruned. On the foliage and stems, the incidence of necrosis was assessed. Necrosis on the stem did not occur, but premature senescence of the leaves observed as chlorosis and necrosis (Table 3) was prevalent in the trial, mainly between 3 and 9 WPI. Again, plants inoculated with the CH2-aggressive

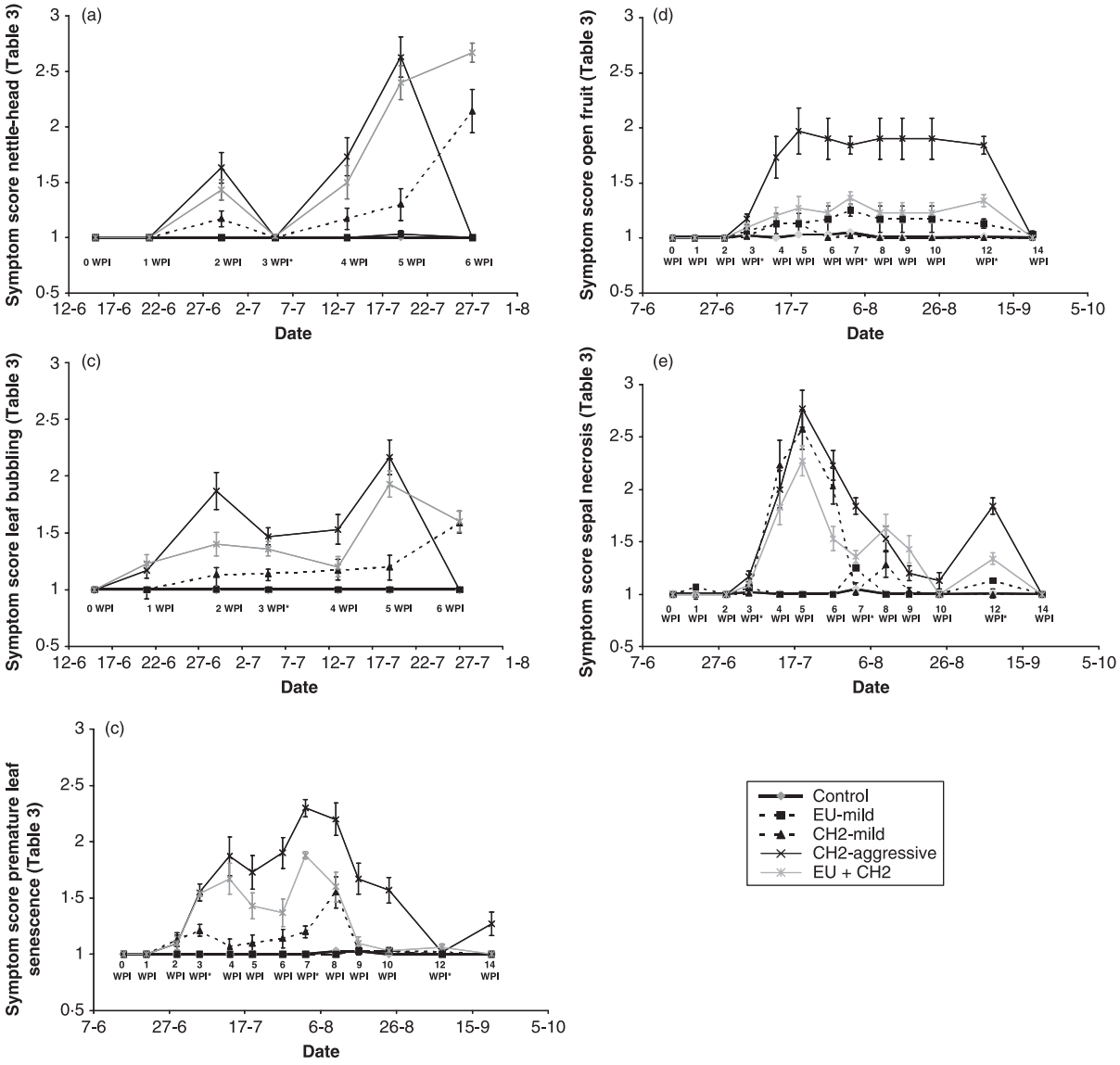


Figure 2 *Pepino mosaic virus* (PepMV) symptom expression on tomato, presented as the evolution of symptom scores (Table 3) in tomato plants inoculated with different PepMV isolates, throughout the trial period. Plants were inoculated with PepMV on 13 June. Each point represents the mean of 30 scores obtained from 30 plants at a certain time point, indicated using the number of weeks post-inoculation (WPI) and the actual date on the horizontal axis. Time points marked with * represent the means of 100 scores obtained from 100 plants. Standard errors bars are indicated for each measurement. (a) Nettle-head (scores given until the end of July, when plants were topped); (b) leaf bubbling in the head of the plants (scores given until the end of July, when plants were topped); (c) premature leaf senescence (necrosis and/or chlorosis); (c) open fruits; (e) browning of sepals.

isolate or the EU + CH2 isolate exhibited the most severe symptoms, with significantly ($P < 0{\cdot}05$) more premature leaf senescence throughout the trial period, symptoms that were not observed in the EU-mild-inoculated plants or the mock-inoculated control (Fig. 2c).

With regard to fruit quality, scores were given for fruit marbling, fruit flaming or blotchy ripening, incidence of scars and open fruits, and necrosis or browning of the sepals (Table 3). In weeks 9, 13 and 15 all ripe tomatoes were rated for fruit marbling (Fig. 3a). Marbling was most prevalent in the EU + CH2-inoculated plants, with almost 10% of the ripe tomatoes displaying severe marbling at 13 WPI. While fruit marbling is a very specific virus-related symptom, flaming of tomatoes is a problem that can also be induced by environmental conditions in an otherwise healthy crop. In this trial, PepMV infection significantly increased the number of flamed or discoloured tomatoes (Fig. 3b), especially for the CH2-aggressive isolate. An open fruit is defined as a fruit that splits shortly after setting, so that the seeds are visible in the flesh of the mature tomato (Table 3). From 4 WPI onwards, the incidence of scars and open fruits was significantly ($P < 0{\cdot}05$

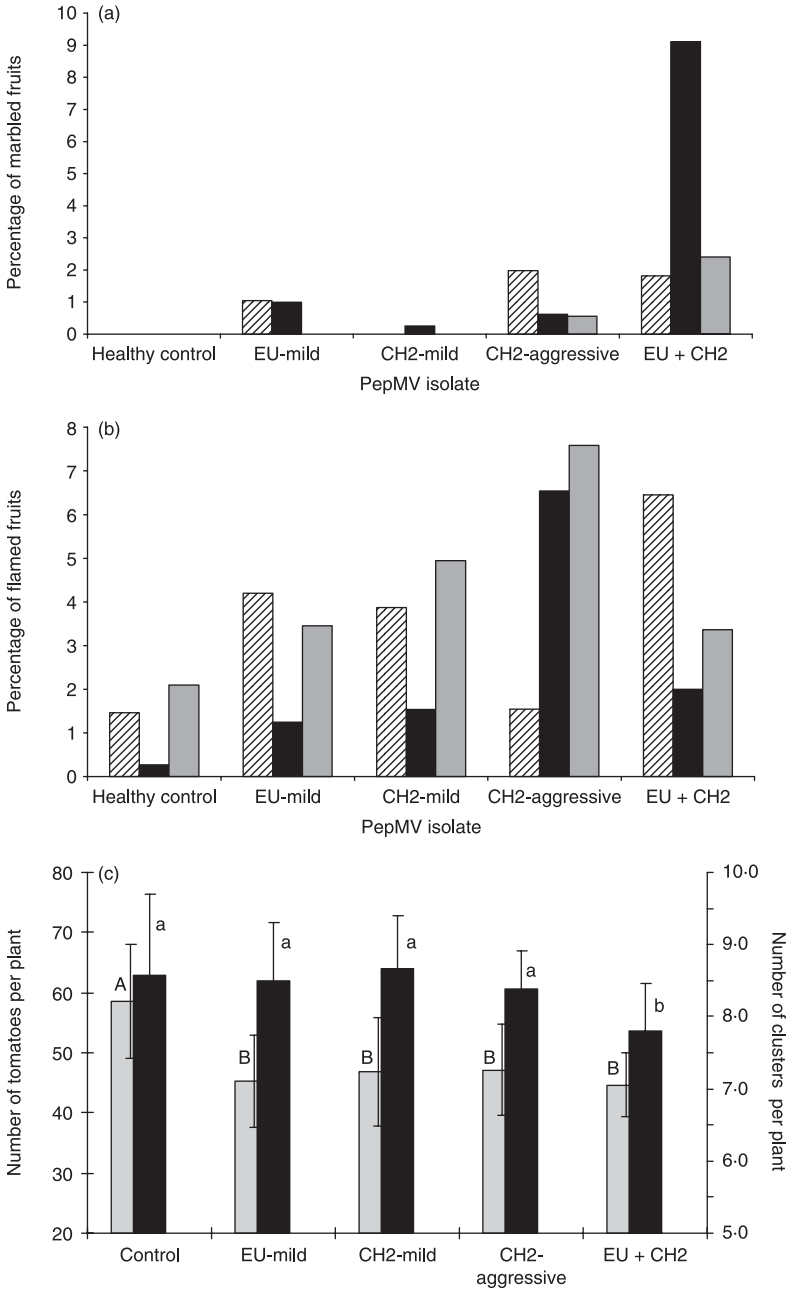


Figure 3 *Pepino mosaic virus* (PepMV) effects on tomato quality and fruit setting, caused by different PepMV isolates. Percentage of tomatoes showing (a) marbling and (b) flaming (discoloration and blotchy ripening) at three different time points. The time point 9 weeks post-inoculation (WPI) is shown in diagonally striped bars; 13 WPI is shown in dark grey bars; and 15 WPI is shown in light grey bars. (c) Fruit setting: total number of tomatoes (left vertical axis, light grey bars) and clusters (right vertical axis, dark grey bars) per plant for the four different inoculations and the control, over the entire growing period. Statistical differences are indicated with a and b for the number of clusters and with A and B for the number of tomatoes (one-way ANOVA, post-hoc Bonferroni, $P < 0{\cdot}05$). Error bars represent standard errors.

at all sampling points from 4 to 12 WPI) higher in plants inoculated with the CH2-aggressive isolate than in the other treatments (Fig. 2d). In these plants, the overall percentage of clusters with at least one open fruit was 10%, compared with 4% and 3% in plants inoculated with EU + CH2 and EU-mild, respectively.

In this trial it was clearly shown that PepMV can cause sepal necrosis (Table 3), a symptom that is not typically associated with PepMV infection, but that radically reduces the commercial value of cluster tomatoes. A high incidence was recorded in plants infected with the CH2 isolates and in the mixed infection, with the CH2-aggressive treatment resulting in the highest score at 5 WPI, followed by the CH2-mild and EU + CH2 treatments (Fig. 2e). No sepal necrosis was seen in the control treatment or in the EU-mild treatment.

Plant vigour and yield were assessed by counting the number of tomato clusters, as well as the total number of tomatoes per plant. Plants in the mock-inoculated control produced significantly more fruits per cluster than plants inoculated with the four PepMV isolates (Fig. 3c). No significant differences were seen between plants inoculated with the EU-mild, CH2-mild and CH2-aggressive isolates. However, the number of clusters per plant was significantly lower for plants inoculated with the EU + CH2 isolate (Fig. 3c).

Table 4 Amino acid polymorphisms in the predicted protein sequences of *Pepino mosaic virus* isolates CH2-mild and CH2-aggressive

	Position[a]	1906 CH2-mild	PCH 06/104 CH2-aggressive
ORF1	504	Glutamic acid	Alanine
	995	Valine	Isoleucine
	1051	Threonine	Serine
ORF2	154	Valine	Alanine
	192	Serine	Proline
ORF3	97	Serine	Asparagine
ORF4	24	Alanine	Threonine
ORF5	48	Threonine	Isoleucine
	244	Alanine	Threonine

[a]Distance from the first amino acid of the protein.

Overall, it was concluded that the CH2-aggressive and EU + CH2 isolates caused significantly more severe symptoms than the EU-mild and CH2-mild isolates.

Comparison of PepMV whole genome sequences

Whole genome sequences, except for the 5′ and 3′ untranslated regions (UTR), were determined for the three single isolates EU-mild, CH2-mild and CH2-aggressive. Several primer sets targeting seven partially overlapping regions of the PepMV genome were used for sequence determination of a total of 6291 nt of the PepMV genome (Table 2). While sequence homology between the EU-mild isolate and both CH2 isolates (CH2-mild and CH2-aggressive) was only 79%, as could be expected (Ling, 2007; Hanssen *et al*., 2008), homology between the two CH2 isolates was as high as 99·4%, the sequences differing only in 38 single nucleotide polymorphisms (SNPs). Collectively, these SNPs cause only nine amino acid differences in the respective predicted proteins between the two CH2 isolates: three in ORF1, two in ORF2, one in ORF3, one in ORF4 and two in ORF5 (Table 4). The UTR between ORF1 and ORF2 (25 nt) was highly conserved between the PepMV isolates, with only one SNP between the EU and the two CH2 isolates and complete homology between the latter two, while the size of the UTR between ORF 4 and ORF5 varied between the EU-mild isolate (38 nt) and both CH2 isolates (45 nt). A single SNP was found in this region when comparing the CH2-mild and the CH2-aggressive isolate. Sequencing of 10 clones obtained from the 625-bp RdRp fragment of the EU + CH2 isolate revealed the presence of three genotypes, including the EU tomato genotype, the CH2 genotype and a recombinant genotype.

Variation of partial PepMV genome sequences

The variation of a 625-bp fragment of the replicase gene and the first ORF of the TGB was studied for the EU-mild, CH2-mild and CH2-aggressive isolates throughout the trial period. Nucleic acid sequences obtained from samples taken at 1, 3, 12 and 16 WPI were compared (Table 1). For all three isolates, minor differences were observed between the sequences obtained from different clones and at different time points. In isolate EU-mild, only silent mutations were observed (data not shown). All sequences from this isolate obtained after 3 WPI differed in one base pair (position 348: A to C substitution) from the sequences obtained at 1 WPI, suggesting that a stable but silent point mutation had occurred. Similarly, sequences obtained from both CH2 isolates displayed a number of SNPs at different time points, although most of the point mutations were again silent (Fig. 4). For both CH2 isolates, missense point mutations, leading to amino acid changes, were also observed (Fig. 4). None of these mutations in the studied genome region were stable, as they disappeared by 16 WPI.

Discussion

Greenhouse trials were conducted to compare the symptoms caused by four different PepMV isolates, originally isolated from four different commercial Belgian tomato crops which differed considerably in PepMV symptom display. As the impact of environmental growth conditions and tomato genotype on PepMV symptom development is not yet fully understood, it was not clear whether the differences in symptom display in these commercial tomato greenhouses should be attributed to the viral isolate, or to environmental factors and cultural practices. In this study, the environmental conditions and plant genotype were standardized in order to study the impact of the viral isolate. The analysis strongly suggested that the viral isolate largely determines symptom development. The EU isolate 1806 (EU-mild) and the CH2 isolate 1906 (CH2-mild), which caused only mild symptoms in the crops of origin, caused rather mild symptoms in the present analysis, while the CH2 isolate PCH06/104 (CH2-aggressive) and the mixed infection isolate 0506 (EU + CH2) that originated from two different tomato crops with severe PepMV symptom display, resulted in the most severe PepMV damage here. Generally, the CH2-aggressive isolate caused the most severe fruit and leaf symptoms throughout the trial period, followed by the EU + CH2 isolate. The latter caused considerably more fruit marbling than all the other isolates.

Interestingly, significant differences in symptom severity were recorded for isolates belonging to the same genotype. The occurrence of open fruits appeared to be associated with the CH2-aggressive isolate. This isolate was clearly more aggressive than the CH2-mild isolate, although only minor differences were found in their genome and amino acid sequences. A total of 38 SNPs was found in the 6291-nt sequence that was determined when comparing both isolates, resulting in only nine differences at the predicted amino acid level. The SNPs were not concentrated in a specific region of the genome. These results confirm the hypothesis that minor differences at the nucleotide level can account for biological differences between isolates. A comparative study using test plants with 14 EU tomato isolates, displaying 99·1–100% nucleic acid sequence homology in a 547-nt fragment of the replicase gene,

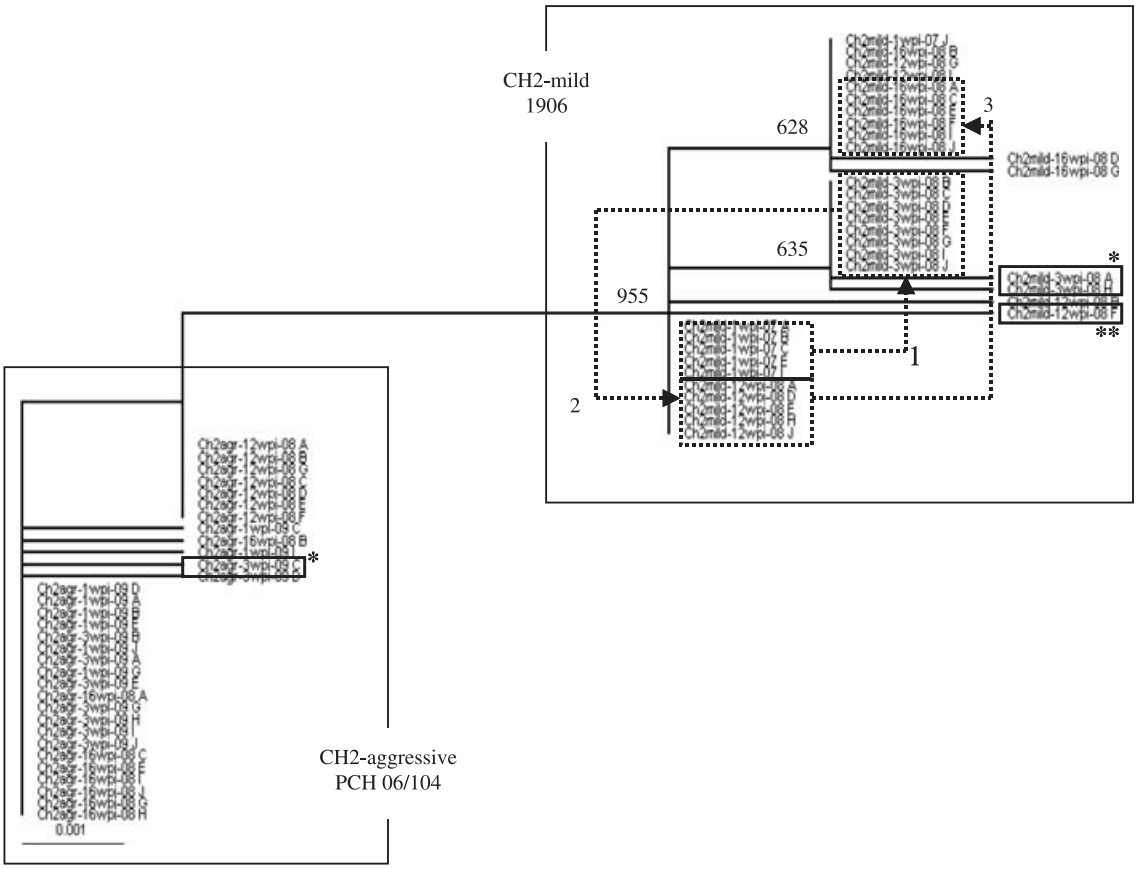


Figure 4 Sequence variation of *Pepino mosaic virus* isolates CH2-mild and CH2-aggressive, presented as a neighbour-joining tree of 67 sequences obtained from samples taken at four different time points. Sequence identifiers contain information about the PepMV isolate used, the sampling time point and the sampling block, separated by hyphens. Predicted translated sequences are identical, except where marked with * (one amino acid substitution) or ** (two amino acid substitutions). Dotted arrows indicate reversible mutations in the CH2-mild isolate (1: 1–3 weeks post-inoculation (WPI); 2: 3–12 WPI; 3: 12–16 WPI).

revealed minor biological differences upon inoculation of test plants (Verhoeven *et al.*, 2003). Comparison of the whole genome sequence of two EU tomato isolates differing slightly in symptom expression revealed 99% homology at the nucleotide level (López *et al.*, 2005). Nevertheless, it remains unclear which regions of the PepMV genome are involved in the expression of symptoms.

ELISA analyses revealed a slower colonization of the tomato crop by the EU-mild isolate than by the other isolates. As plants were inoculated on the lower leaves and samples were taken from the upper, young leaves, the virus could only be detected after efficient systemic movement. Therefore, the slower colonization could be the result of impeded phloem-dependent accumulation of this mild PepMV isolate, as was also observed for the attenuated M strain of *Tobacco mosaic virus* (TMV) (Nelson *et al.*, 1993). The mixed infection resulted in the highest and most stable titres throughout the entire period, while the viral titre in plants inoculated with the EU-mild isolate was very unstable and generally the lowest. As viral synergism is often manifested by an increase in both symptom expression and viral accumulation (Hull, 2002), the higher titre observed in plants infected with the EU + CH2 isolate could indicate a synergistic interaction between the coinfecting EU and CH2 genotypes, but further research is needed to confirm this hypothesis.

The results suggest a correlation between the aggressiveness of the PepMV isolate and the viral titre. Interestingly, a lower PepMV titre in symptomless tomato plants infected with a Peruvian genotype isolate (isolate LP 2001) compared to tomato plants infected with EU tomato genotype isolates showing clear PepMV symptoms was previously reported (López *et al.*, 2005). A lower viral titre may occur for viral isolates with reduced post-transcriptional gene silencing (PTGS) suppressor activity, as PTGS reduces viral accumulation (Ratcliff *et al.*, 1999). PTGS has been associated with differential symptom expression, viral resistance and synergism of viruses in previous studies (Pruss *et al.*, 1997; Ratcliff *et al.*, 1999; Kubota *et al.*, 2003).

It may be expected that the PepMV genome displays high mutation frequencies, as many different genotypes have emerged over a rather limited period of time. In general, RNA virus replication is characterized by high

mutation rates, high yields and short replication times (Domingo & Holland, 1997). Therefore, RNA sequence stability of the PepMV isolates used in the present greenhouse trails was studied by comparing sequences of a 625-nt fragment, containing the end of the replicase gene, an untranslated region and the start of the TGB, obtained from samples taken at four time points throughout the trial period. Comparison of sequences obtained from the beginning and end of the trial period revealed that the number of mutations was rather limited and that most of the mutations that took place had no clear biological relevance, as they were mostly silent and often reversible. Therefore, the RNA sequence in this part of the genome appeared to be relatively stable. These results are compatible with recent advances in plant virus evolution on random genetic drift and the existence of critical thresholds that limit viruses to a small portion of their potential sequence space (Domingo & Holland, 1997). It was shown that systemic movement of plant viruses through the vascular system results in population bottlenecks. A study on systemic movement of TMV revealed that effective population numbers were much smaller than census population numbers, indicating the importance of random genetic drift in virus evolution (Sacristan *et al.*, 2003). Deleterious mutations can lead to average fitness losses, thus restricting the types and numbers of mutations RNA viruses can tolerate (Domingo & Holland, 1997; Garcia-Arenal *et al.*, 2001).

A high stability of the RNA sequence of the PepMV genome is also in line with the observation that sequence homology of different isolates from different origins but belonging to the same PepMV genotype groups show high levels of sequence homology (> 99%) (Verhoeven *et al.*, 2003; López *et al.*, 2005). However, it remains unclear how the variability in currently known PepMV genotypes was introduced in the viral genome.

This is apparently the first study in which differential symptom display in greenhouse tomato production has been unambiguously attributed to the PepMV isolate. These results can at least partially explain the huge variation in the level of damage reported for PepMV in commercial tomato production. A recent report on the biological characterization of several PepMV isolates on solanaceous test species in climate chambers (Córdoba-Sellés *et al.*, 2008) confirmed the finding that different PepMV isolates display differential pathogenic behaviour. In addition, the present study identified a number of SNPs that may play a role in PepMV symptom expression. These results might contribute to future identification of genome regions involved in the expression of PepMV symptoms in tomato.

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