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The use of attenuated isolates of *Pepino mosaic virus* for cross-protection

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Abstract Pepino mosaic virus (PepMV) has recently emerged as a highly infectious viral pathogen in tomato crops. Greenhouse trials were conducted under conditions similar to commercial tomato production. These trials examined whether tomato plants can be protected against PepMV by a preceding infection with an attenuated isolate of this virus. Two potential attenuated isolates that displayed mild leaf symptoms were selected from field isolates. Two PepMV isolates that displayed severe leaf symptoms were also selected from field isolates to challenge the attenuated isolates. The isolates with aggressive symptoms were found to reduce bulk yields by 8 and 24% in single infections, respectively. Yield losses were reduced to a 0–3% loss in plants that were treated with either one of the attenuated isolates, while no effects were observed on the quality of the fruits. After the challenge infection, virus accumulation levels and symptom severity of the isolates with aggressive symptoms were also reduced by cross-protection. Infection with the attenuated isolates alone did neither affect bulk yield, nor quality of the harvested tomato fruits.

Keywords Attenuated isolate · Cross-protection · *PepMV* · Tomato

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Introduction

Tomato *(Solanum lycopersicum)* is one of the world's most widely grown vegetables. Tomato is susceptible to various viral diseases, and one of the causal agents, *Pepino mosaic virus* (PepMV), has recently become a major limiting factor with regard to tomato production.

After its initial discovery on pepino (*Solanum muricatum*) in Peru, PepMV was not reported until it was rediscovered in tomato crops in Europe in 1998/1999 (Van der Vlugt et al. 2000; Soler-Aleixandre et al. 2005). Ever since, PepMV has rapidly spread throughout greenhouse tomato production and is currently found throughout Europe and North-America (Jorda et al. 2001; Cotillon et al. 2002; Verhoeven et al. 2003; Ling et al. 2008). The RNA genome of PepMV encompasses approximately 6.4 kb and contains five open reading frames that encode an RNA-dependent polymerase (RdRp), a triple gene block (TGB), a coat protein (CP), and two short untranslated sequences flanking the coding regions (Aguilar et al. 2002; Cotillon et al. 2002).

Based on sequence similarity, PepMV isolates are grouped into four separate strains, namely the Peruvian (PE)-strain to which the original PepMV isolate belongs, the European (EU)-strain that was found in Europe in 1999 (Van der Vlugt et al. 2000), the CH2-strain that was discovered in infected tomato seeds in Chile, and the US1-strain that was discovered in diseased tomato plants in the USA (Ling 2007). Symptom severity varies between different isolates of PepMV (Van der Vlugt et al. 2000) and differences in severity do not necessarily coincide with differences in genotype (Hanssen et al. 2007).

PepMV induces a wide range of symptoms on tomato, such as mosaic, leaf distortion, nettle-like heads, single yellow spots, inter-veinal chlorosis, and fruit discolouration (Van der Vlugt et al. 2000; Jorda et al. 2001). Tomato plants display symptoms shortly after infection with PepMV and, in general, symptoms subsequently subside (Van der Vlugt and Stijger 2008). However, symptoms may return later during the growing season. Expression of symptoms may also depend on environmental conditions, such as temperature and light intensity (Jorda et al. 2001; Van der Vlugt and Stijger 2008). PepMV is sometimes suggested to cause yield losses in tomato, but the highest economic losses are attributed to symptoms that affect the commercial value of tomato fruits, such as flaming, marbling, blotchy ripening, and fruit size reduction (Soler et al. 2000; Spence et al. 2006). Striking differences in the severity of symptomology have been reported earlier (Verhoeven et al. 2003) and not all isolates cause typical PepMV symptoms such as marbled and flamed fruits (Hanssen et al. 2009).

PepMV is transmitted very efficiently by contaminated hands, clothing or tools (Van der Vlugt and Stijger 2008). Direct contact between healthy and infected plants during routine crop handling also suffices to spread PepMV infection. Strict hygiene measures are required to prevent infections and to limit the spread of PepMV in greenhouses. The incidence of PepMV is very high in some tomato cultivation areas, where the virus may affect up to 90% of the tomato producing greenhouses (Soler-Aleixandre et al. 2005). Staying free of PepMV is challenging under these circumstances. Cross-protection may prove to offer an alternative strategy to reduce economic losses. The principle of cross-protection describes the phenomenon of protecting crops against virulent isolates of viruses by pre-treatment with closely related attenuated isolates of the virus. Cross-protection has been applied to control various viral diseases. In the 1970s, the mild MII-16 isolate was used successfully to control infections of tomato with *Tobacco mosaic virus* in several countries (Burgyán and Gáborjányi 1984). Other examples include the use of attenuated variants of *Citrus tristeza virus* (Costa and Muller 1980), *Papaya ringspot virus* (Yeh et al. 1988), *Chinese yam necrotic mosaic virus* (Kondo et al. 2007), *Pepper mild mottle virus* (Yoon et al. 2006), and *Cucumber mosaic virus* (Kosaka and Fukunishi 1997). Attenuated isolates have either been selected among naturally occurring isolates (Costa and Muller 1980; Kondo et al. 2007) or have been developed by the introduction of mutations into wild type isolates (Yeh et al. 1988).

PepMV can have a serious economic impact on the tomato production. Cross-protection may prove to be an effective control measure. This strategy relies on the availability of an attenuated isolate that effectively protects against more virulent isolates, while having as little impact on total yield and fruit quality as possible. In this study, we show that the use of attenuated PepMV isolates under greenhouse conditions can reduce the detrimental effects of two PepMV isolates which cause severe foliage symptoms and yield reductions in a single infection. The effects of the attenuated isolates on bulk yield were minimal, while no fruit symptoms were observed.

Materials and methods

PepMV isolates

Four PepMV isolates were selected on the basis of their known virus-associated symptoms in tomato. The isolates *EU-Att1 (=PD99901066)* (Van der Vlugt et al. 2000; Verhoeven et al. 2003) and *PE-Att1* were used as attenuated isolates. The isolates *EU-Chl1* and *EU-Nec1* were used as challenging isolates. All isolates have been characterized on experimental host plants *(Nicotiana glutinosa*, *N. occidentalis*, *Chenopodium quinoa*, and *Solanum lycopersicum)* and inocula were tested by both Enzyme-linked immunosorbent assay (ELISA) and Polymerase Chain Reaction (PCR)-based methods to ensure the absence of other tomato viruses known to occur in the Netherlands. *EU-Att1* was the first PepMV isolate

that was detected in the Netherlands (Van der Vlugt et al. 2000; Verhoeven et al. 2003). Its full-length sequence has been determined (GenBank FJ940223). *EU-Nec1* was collected in a commercial greenhouse in 2003. The full-length sequence of *EU-Nec1* has also been determined (GenBank FJ940224). *EU-Nec1* causes necrotic symptoms on leaves, stems and crowns of the fruits (Fig. 3). *PE-Att1* and *EU-Chl1* were both collected from commercial greenhouses in the west of the Netherlands in 2004. *Pe-Att1* sometimes causes mild mosaic, single yellow spots on the leaves and mild leaf bubbling in the heads of tomato plants. Symptoms caused by *EU-Chl1* include severe chlorosis. Partial sequences of *PE-Att1* and *EU-Chl1* have been determined (GenBank).

Greenhouse trials

Trials were carried out at the greenhouse facilities of Applied Plant Research in Naaldwijk (The Netherlands) in 2006. Plants of the tomato cultivars *Cedrico* (medium tomato on the vine, Rijk Zwaan), *Ever* (large tomato on the vine, De Ruiter Seeds) and *Ingar* (large loose tomato, Enza Zaden) were grown in identical greenhouse compartments. All cultivars are common commercial cultivars in the Netherlands. Both grafted and non-grafted plants of the three cultivars were tested. The tomato cultivar *Maxifort* was used as rootstock. Tomato seedlings were sown in rockwool plugs and transplanted into single rockwool blocks that were subsequently placed on rockwool slabs. Tomato plants were trained to a crop wire at 3.5 m height.

Compartments had a cropped area of 186 m^2 and encompassed 2×3 parallel experimental plots that each contained a double row of plants. Treatments were separated by rows of sweet pepper (*Capsicum annuum*, cv. *Spider*), which is considered a non-host for PepMV. Absence of PepMV in these plants was confirmed by ELISA. A single row of tomato guard plants was planted parallel to the experimental plots along the walls of the compartments to avoid end effects. No guard plants were located transverse of the plots, because all plants are rotated in a carousel and therefore had the same exposure to edge effects. Replicate plots were located within the same compartment because of the high risk for contamination between treatments. Each plot consisted of 36 tomato plants.

All treatments were grown under the same standardized environmental conditions. Tomato plants were maintained at temperatures regimes that were comparable to regimes applied in commercial tomato production in the Netherlands. Environmental conditions (temperature, light intensity, relative humidity, and CO_2 concentration) were recorded by data loggers. Treatment with insecticides and fungicides was required to control thrips, whiteflies, spider mites, caterpillars, and *Botrytis*.

The trials encompassed nine treatments, namely a virus-free control treatment, four control treatments of each isolate in a single infection, and four cross-protection treatments. Virus preparations were revived on living tomato plants before application in the greenhouse trials. Crude sap from the upper leaves was extracted in inoculation buffer (PBS, pH 7.4) at a dilution of 1:10 (w/v). PepMV concentrations were determined in inoculation source material by DAS-ELISA prior to inoculations. Inocula were adjusted with sap from healthy leaves to ensure that identical amounts of virus were present. Inoculations with the attenuated isolates were conducted onto carborundum-dusted leaves in 6th leaf-stage tomato plants on January 12, 2006. Six weeks later, plants were challenged with *EU-Nec1* and *EU-Chl1* on February 23, 2006. Control treatments of single infections with the challenge isolates were also inoculated on February 23, 2006. Inoculations were performed on the uppermost fully developed leaves.

A very strict hygiene protocol was implemented to avoid contaminating infections between treatments. Each treatment had its own stock of equipment, overalls, gloves, shoe covers and caps. Equipment of different treatments was marked by different colours to avoid confusion. Disinfection mats were placed in front of all entrances and were wetted regularly. All personnel followed a specific working-order to reduce the consequences of accidental contamination between treatments. The uninfected treatment was always visited prior to the other treatments. The treatments with single infections of the attenuated isolates were visited next, followed by the cross-protection treatments and the treatments with single infections of the challenge isolates. The same personnel, as instructed, performed all labour throughout the growing season. As bumble bees can spread PepMV between treatments (Shipp et al. 2008), they were not introduced into the trial

and pollination was done by hand. Introduction of insects from outside was avoided by screening on the glasshouse vents, and no biological control was applied.

DAS-ELISA

Double sandwich enzyme-linked immunosorbant assay (DAS-ELISA) was used to monitor the absence of PepMV in non-infected control plants and to determine the total PepMV concentration in infected plants. The uppermost fully developed leaves were sampled from five plants per treatment weekly till March and monthly afterwards, adding up to a total of 16 samples per plant. Leaf samples were stored at –80°C until they were simultaneously analyzed at the end of the season. Leaf tissue was diluted 1:10 (w/v) in PBS-Tween buffer (pH 7.4) and ground. Anti-PepMV rabbit polyclonal antiserum (Prime Diagnostics, The Netherlands) was used to determine PepMV concentrations. This antiserum has been raised against the EU strain of the virus, but given the high similarity between the CP of the EU and PE-strain (100%) no differences in reactivity are expected with regard to reactivity to the PE-strain. The antiserum was diluted to 1:1,000 (v/v) in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$ and 0.003 M NaN_3, pH 9.6) and incubated on microtitre plates at 37°C. After two hours, the plates were washed in PBS-Tween and sap extract was added to the wells. Plates were subsequently incubated at 37°C for two hours and again washed in PBS-Tween. Alkaline phosphatase-conjugated anti-PepMV polyclonal antiserum was diluted 1:1,000 in PBS-Tween (pH 7.4) and incubated at 37°C for two hours. After a final washing step, substrate (p-nitrophenyl phosphate disodium) was added at 1 mg/ml in substrate buffer (1 M diethanolamine and 0.003 M NaN_3, pH 9.8). Reactions were measured at 405 nm with an ELISA reader (Bio-Tek ELx808) after incubation at room temperature for one hour. Samples were considered positive if optical density values exceeded the mean background level by a factor three. Mean background levels were determined for each ELISA plate by measuring at least two wells that contained all reagents except the sap extract. To determine viral concentrations, a dilution series of known amounts of PepMV was assayed on each plate to establish a standard curve.

RT-PCR

Specific primers were developed to identify the individual isolates of PepMV that were used in these trials. Total RNA was extracted from tomato leaves using Plant RNA reagent (Invitrogen) according to the manufacturers' instructions. cDNA was synthesized using AMV reverse transcriptase (Sigma-Aldrich). We identified base substitutions that are specific for *EU-Att1* and *PE-Att1* in the RdRp gene, and for *EU-Chl1* and *EU-Nec1* in the triple gene block, and designed specific primers (Primer Express, Applied Biosystems) for the RT-PCR. Primes used to distinguish these variants were: *EU-Att1*-F 5′-CCTCCCGACCCAGTGGATTTC-3′ and *EU-Att1*-R 5′-GGGAAATTTTGTTAGCGTCG-3; *PE-Att1*-F 5′-CGCATATCAACATGTTCGACCC-3′ and *PE-Att1*-R 5′-GTGTGTTTGGATTGCGTGGGGA-3′; *EU-Chl1*-F 5′- CAAAAAGATATCTTATTTTCCACA ACAA-3′ and *EU-Chl*-R 5′- TGTTGGTTGATGA ATGTGTGTTG-3; *EU-Nec1*-F 5′-GAATTCTT TAACCCTTTTGAAGTG-3′ and *EU-Nec1*-R 5′-AGTGGTCACCACTTGGTCAGAG-3′. PCR amplification with each primer pair was performed in 25 µl reactions containing SYBR GREEN PCR Master Mix (Applied Biosystems). PCR products were sequenced to check the identity of the fragments.

Symptom development, harvest and quality assessment

Occurrence of symptoms was monitored using a standardized scoring form on which the apical leaves and foliage were rated. Symptoms were recorded once a week and all treatments were assessed on a single day. Observed symptoms in the apical leaves were leaf bubbling, nettle heads, leaf necrosis, stem necrosis, and mosaic. Observed foliage symptoms were yellow spots, leaf necrosis, stem necrosis, leaf distortion, and chlorosis. Each symptom was scored on a 0 (no symptoms) to 3 (very severe) scale (Table 1). For each treatment, five plants were evaluated per cultivar for both grafted and non-grafted plants.

Tomato fruits were harvested once a week from April till September for a total of 24 weeks. On the day of harvest, fruits were rated for the presence of marbling, fruit discolourations (marbling, flaming, blotchy ripening), and blossom-end rot. Total weekly harvest was assessed per plot per cultivar for both grafted and non-grafted plants.

Table 1 PepMV related symptoms on tomato that were recorded during the trials

Plant part	Symptom	Score	Description of symptom severity	Number of times observed[a] (N=8304)
Apical leaves (upper 3 compound leaves)	Leaf bubbling	1	Bubbled surfaces on some (single) leaves	401
		2	Majority of leaves bubbled	35
		3	All leaves bubbled	1
	Nettle heads	1	Leaves with a slightly reduced leaf area, slightly pointed	1162
		2	Leaves with a reduced leaf area, pointed	153
		3	Nettle-like leaves	38
	Leaf necrosis	1	Individual necrotic spots on some single leaves	191
		2	Majority of compound leaves have necrotic spots	89
		3	Majority of single leaves have necrotic spots	30
	Stem necrosis	1	One necrotic streak	45
		2	Multiple necrotic streaks	27
		3	Necrotic streaks > 2 cm	1
	Mosaic	1	Slight discoloration	5454
		2	Clear discoloration	1242
		3	Mosaic	46
Foliage	Yellow spots	1	One or some yellow spots	386
		2	Majority of compound leaves contain yellow spots	5
		3	All compound leaves contain yellow spot(s)	4
	Leaf necrosis	1	Necrotic areas on some single leaves	623
		2	Majority of leaves with necrotic areas	366
		3	Majority of leaves with >50% leaf area affected	241
	Stem necrosis	1	One necrotic streak	126
		2	Multiple necrotic streaks	100
		3	Necrotic streaks >5 cm present	3
	Leaf distortion	1	Some leaves irregularly shaped	490
		2	Some leaves with a reduced leaf area	175
		3	Majority of leaves with a reduced leaf area	135
	Chlorosis	1	Some leaves slightly chlorotic	50
		2	Some leaves strongly chlorotic	49
		3	Majority of leaves chlorotic	5

[a] Across all treatments

Statistical analysis

All statistical procedures were performed in SPSS 15.0. Virus concentrations and symptoms were subjected to a repeated measures mixed linear model. This procedure can be applied to analyze longitudinal multilevel data with missing data (caused by drop outs). Models were fitted using the residual maximum likelihood (REML) algorithm. A step down test was performed (significance level at p=0.05) to arrive at a well-fitting covariance structure using the deviance values of the covariance matrices. The "unstructured" matrix was retained for the analysis. The study focused on differences between PepMV treatments. Therefore, “Cultivar” was included as a covariate in all analyses to reduce the complexity of interpreting the results. When applying a repeated measures analyses, the effect sizes of differences between host cultivars on the variables virus concentration and symptoms were small compared to effect sizes of differences between PepMV treatments (h_p^2=0.545 vs. 0.004, and h_p^2=0.479 vs. 0.008, respectively). A

repeated measure was used to test differences in yield and fruit quality. *Post-hoc* Bonferroni tests were used to test differences between groups. Tests for correlation analysis (Spearman's rho) were also performed using SPSS.

Results

Presence of PepMV

All tomato plants were free of PepMV before inoculation as determined by DAS-ELISA. The non-infected controls remained virus free throughout the trials. One week after inoculation with the *EU-Att1* isolate, this PepMV isolate was detected by ELISA in all inoculated plants. All plants inoculated with *PE-Att1* were ELISA positive four weeks after inoculation.

RT-PCR was used to determine whether the inoculations with the isolates (*EU-Att1, PE-Att1)* were successful and whether the challenge isolates became established *(EU-Chl1,* and *EU-Nec1)*. The presence of the challenge isolates in the "cross-protection" treatments was confirmed three weeks after the second round of inoculations. The occurrence of accidental contamination between different treatments was also monitored by RT-PCR on a monthly basis. No accidental contaminations occurred between any of the treatments.

Virus concentration

Total PepMV concentrations were determined by DAS-ELISA. They fluctuated throughout the growing season depending on the isolates that were present in the plants (Fig. 1). Virus accumulation levels differed significantly between plants grown in different treatments ($F(8,207)=213.91$, $p<0.001$). Grafting had no effect on PepMV concentration ($F(1,256)=0.15X$, $p=0.70$) and no significant interaction between treatment and grafting ($F(8,210)=0.46$, $p=0.88$) was observed.

Virus accumulation was significantly lower in plants that were infected with a single infection of the attenuated isolates compared to plants infected with a single infection of the challenge isolates. This difference amounted on average to a 14 to 17 times lower PepMV concentration in the plants infected with the attenuated isolates. The PepMV concentration of the single infections with the attenuated isolates was low throughout the season and did not reach any pronounced peaks (Fig. 1). The PepMV concentration of the challenge isolates showed a broad peak after inoculation until June. Virus concentrations of the challenge isolates decreased onwards.

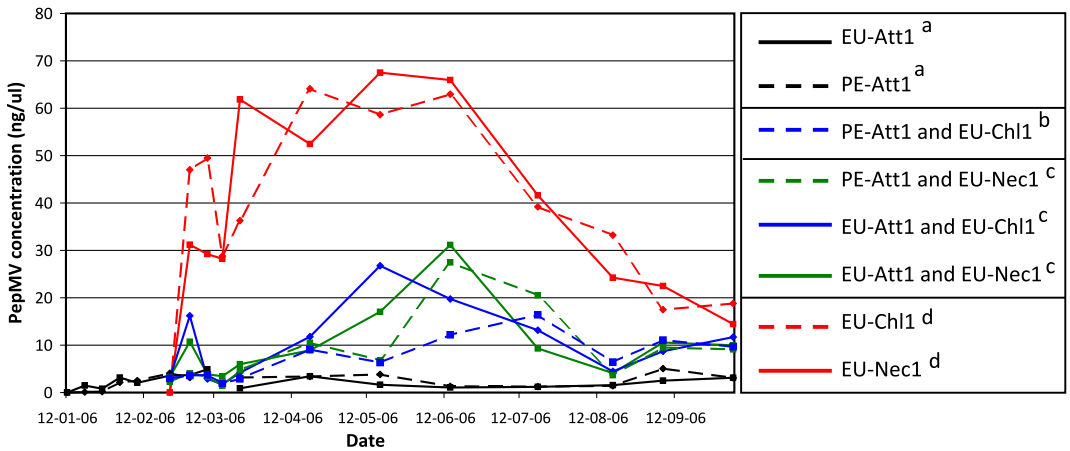

Fig. 1 PepMV concentrations during the growing season as determined by DAS-ELISA. Arrows indicate when the inoculation with the attenuated (1) and challenge isolates took place (2). PepMV concentrations were averaged over grafted and non-grafted plants. A repeated measure mixed linear model was applied to the data taken after the challenge inoculation, and used to evaluate significant differences between treatments. Superscript numbers in the legend indicate treatments that are different at the $p<0.05$ level from other treatments

The cross-protection treatments had intermediate virus concentrations compared to the single infections of attenuated and challenge isolates (Fig. 1). PepMV concentrations in the cross-protected plants were significantly lower than those in the challenged unprotected plants, but were, in turn, significantly higher compared to the single infections of the attenuated isolates. Whereas the PepMV concentration increased rapidly in non-protected plants after infection by the challenge isolates, there was only a small peak directly after inoculation in the cross-protected plants. Then, there was a delayed increase in virus concentration from May to August. The PepMV concentration in the cross-protection treatments was on average three to five times lower compared to the single infections of the challenge isolates.

Symptoms

Both attenuated isolates of PepMV (*EU-Att1* and *PE-Att1*) showed very few symptoms throughout the experiment. Plants that were inoculated with attenuated isolates started to show symptoms within two weeks after inoculation. Both *EU-Att1* and *PE-Att1* caused slight mosaic on the apical leaves of the tomato plants. *EU-Att1* also caused yellow spots on a limited number of plants. Three weeks after inoculation, mosaic symptoms slightly increased and leaf bubbling was observed. Symptoms in plants that were infected with the attenuated isolates became less evident as the season progressed (Fig. 2).

Overall, PepMV-related symptoms differed significantly between plants in different treatments ($F(8,5390)=1929.28$, $p<0.001$). Grafting had no effect on PepMV-related symptoms ($F(1,5390)=0.52$, $p=0.47$X) and there was no significant interaction between treatment and grafting ($F(1,5375)=1.35$, $p=0.25$). The challenging isolates caused significantly more severe symptoms than the attenuated isolates (Table 2). *EU-Chl1*-infected plants started to show various symptoms two weeks after inoculation, including nettle heads, leaf bubbling and slight leaf deformation. Symptoms of the challenge stains also became less evident as the growing season progressed (Fig. 2). Although *EU-Chl1* was classified as aggressive on tomato test plants, the isolate induced relatively mild symptoms. Symptoms of *EU-Nec1* were much more severe throughout the season. Already one week after inoculation, necrotic spots appeared on the inoculated leaves. Two weeks after inoculation, the apical leaves of all plants showed necrosis and mosaic, leaf distortion was present above the inoculated leaves, and necrosis was present on the crowns of developing fruits (Fig. 3). Symptoms subsequently decreased, but neither the necrotic symptoms nor the leaf distortions disappeared completely.

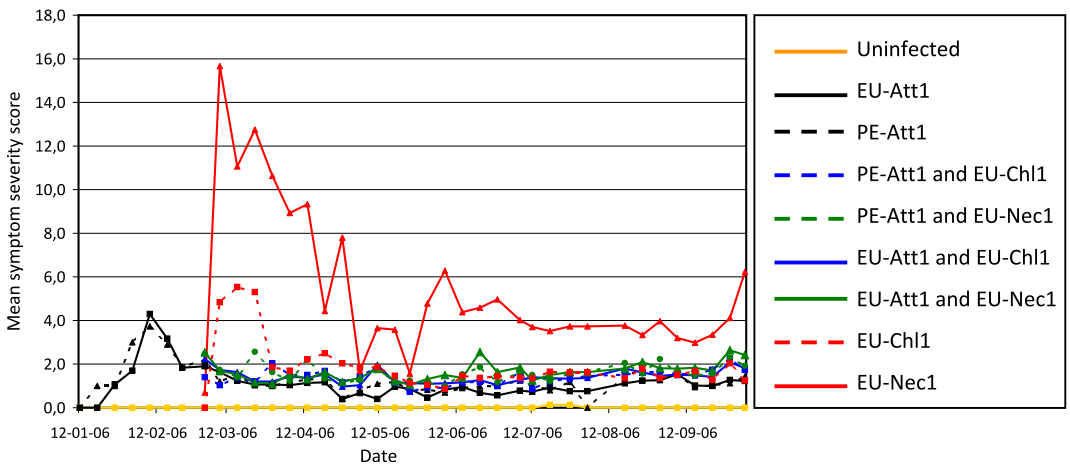

Fig. 2 Severity of the PepMV related symptoms in apical leaves and foliage during the tomato growing season. Displayed symptom scores were averaged over grafted and non-grafted plants. Arrows indicate when the inoculation with the attenuated (1) and challenge isolates took place (2)

Table 2 Average symptom scores throughout the trial[1]

Treatment	Leaf Bubbling	Nettle head	Stem necrosis apical leaves	Leaf necrosis apical leaves	Mosaic	Yellow spots	Leaf necrosis foliage	Stem necrosis foliage	Leaf distortion	Chlorosis	Total symptom severity	Significant differences[2]
Uninfected	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	a
Single infections												
EU-Att1	0.0	0.1	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	1.0	b
PE-Att1	0.0	0.1	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	1.1	b
EU-Chl1	0.2	0.4	0.0	0.0	1.1	0.1	0.0	0.0	0.2	0.0	1.9	d
EU-Nec1	0.1	0.4	0.1	0.4	1.2	0.1	1.1	0.4	1.5	0.1	5.4	e
Cross-protection treatments												
EU-Att1 + *EU-Chl1*	0.0	0.2	0.0	0.0	1.0	0.1	0.0	0.0	0.1	0.0	1.4	c
EU-Att1 + *EU-Nec1*	0.1	0.2	0.0	0.0	1.1	0.1	0.1	0.0	0.1	0.0	1.7	d
PE-Att1 + *EU-Chl1*	0.0	0.2	0.0	0.0	1.1	0.0	0.0	0.0	0.1	0.0	1.4	c
PE-Att1 + *EU-Nec1*	0.1	0.2	0.0	0.0	1.1	0.0	0.1	0.0	0.1	0.0	1.6	d

[1] High numbers indicate more severe symptoms. Each symptom was scored on a 0 (no symptoms) to 3 (very severe) scale and combined to a single "symptom severity" score. Total number of observed plants=270; total number of observation per plant=31. Displayed symptom scores were averaged over grafted and non-grafted plants

[2] Treatments with the same subscript are not significantly different from each other at $p<0.05$.

The symptoms that were induced by the challenge isolates *EU-Nec1* and *EU-Chl1* were significantly reduced in the cross-protected plants compared to the plants infected with a single infection of these isolates (Table 2). Because *EU-Chl1* was not as aggressive as expected, this effect was more evident for *EU-Nec1*. Throughout the growing season, *EU-Nec1* induced more severe symptoms in non-protected plants compared to cross-protected plants (Fig. 2). *EU-Nec1* did induce necrotic symptoms in the cross-protected plants, which appeared about 10 weeks after inoculation at the end of May. After their initial appearance, necrotic symptoms subsided and were less severe compared to the symptoms in plants with a single infection of *EU-Nec1*. However, necrotic symptoms never disappeared entirely. The attenuated isolates *EU-Att1* and *PE-Att1* did not differ with respect to their effectiveness to reduce symptom expression (Table 2).

Effect of PepMV on yield and fruit quality

The first tomato flowers were observed around January 15. Tomato fruits were harvested once a week from April until September. The trial ended on October 5, 2006. Crop management and crop handling was identical in all treatments, except on one occasion in which it was decided to postpone leaf picking in the *EU-Nec1*-infected plants because the young leaves showed severe necrosis and did not develop well. Total leaf area of these plants would have become too low to intercept sufficient light when leaf picking would have been carried out. The remaining leaves enabled the plants to recover considerably.

PepMV did affect total yield of the tomato crop. There was a significant effect of treatment ($F(8,28)=20.88$, $p<0.001$) and of grafting ($F(1,36)=58.98$, $p<0.001$) on yield. Average yield was higher in grafted plants than in non-grafted plants (681±5.9 vs. 615±6.1 g per plant per week), but grafting did not affect the yield reduction that was observed in PepMV infected plants as there was no significant interaction between treatment and grafting ($F(8,36)=1.40$, $p=0.23$). An infection by the necrotic isolate (*EU-Nec1*) caused significantly lower yields compared to the other treatments (Table 3). Table 3 also lists the relative difference in overall yield compared to the

Fig. 3 Symptoms of the *EU-Nec1* isolate. *EU-Nec1* causes necrosis on leaves, stems and the crowns of the fruits

uninfected treatments. Compared to the uninfected treatment, *EU-Nec1* caused an overall yield loss of 24%. When cross-protection was applied before an infection with *EU-Nec1*, overall yield losses improved significantly to a 2% loss. The isolate that caused chlorosis (*EU-Chl1*) caused an overall yield loss of 8%. Cross-protection by *EU-Att1* resulted in a significant improvement and a yield that was no longer significantly different from the healthy control. Cross-protection by *PE-Att1* had no significant effect on the yield loss caused by *EU-Chl1*, although there was a tendency towards reduced yield losses (a 2% loss).

Fruit symptoms such as flamed fruits and marbling were not observed at any time in any of the treatments, including the treatments of the challenge isolates. A limited number of fruits displayed blotchy ripening symptoms, which also occurs when PepMV is absent. The amount of fruits affected by blotchy ripening was neither affected by grafting ($F(8, 36)=1.68$, $p=0.97$), nor by treatment ($F(1, 36)=9.12$, $p=0.22$). No significant interaction was found between treatment and grafting ($F(8,36)=0.06$, $p=1.00$). The only factor which did affect the occurrence of blotchy ripening was time (Fig. 4). The commercial value of fruits is also affected by blossom-end rot, which is not considered to be PepMV related. Blossom-end rot was affected by grafting ($F(1,36)=19.86$, $p<0.001$), but not by treatment ($F(8,36)=0.17$, $p=0.99$). There was also no significant interaction between treatment and grafting ($F(8,36)=0.13$, $p=1.00$). Blossom-end rot occurred more often in non-grafted plants than in grafted plants, namely at 3.57 and 0.89 fruits per plant per week, respectively.

Relation between virus accumulation, symptoms and yield

We observed a positive correlation between virus accumulation and overall symptom severity (Spearman's rho=−0.876, $p<0.0005$). When testing for correlations between the individual symptoms (as mentioned in Table 1) and virus accumulation, all symptoms were significantly correlated except the occurrence of chlorosis. The highest correlation coefficients were observed between accumulation and the occurrence of yellow spots ($\rho=0.738$, $p<0.0005$), nettle heads ($\rho=0.735$, $p<0.0005$) and mosaic ($\rho=0.698$, $p<0.0005$). In turn, overall symptom severity was negatively correlated to yield ($\rho=-0.579$, $p<0.0005$). Here, the strongest negative correlations were found between yield and the occurrence of leaf deformation ($\rho=-0.656$, $p<0.0005$) and leaf necrosis on foliage ($\rho=-0.548$, $p<0.0005$), which is considerably higher than

Table 3 Yield (per week per plant±std. error) and the average number of fruits affected by blotchy ripening and blossom-end rot (per week per plant)[1]

Symptom Treatment	Yield (g per week per plant)	Yield change (%)	[2]	Blotchy ripening (# of fruits per week per plant)		Blossom-end rot (# of fruits per week per plant)	
Uninfected	673±20	reference	a	0.88±0.08	*ns*	0.19±0.04	*ns*
EU-Att1	669±23	−1%	ab	0.86±0.08	*ns*	0.36±0.07	*ns*
PE-Att1	703±23	+ 4%	a	1.12±0.08	*ns*	0.36±0.06	*ns*
EU-Chl1	617±22	−8%	b	0.81±0.07	*ns*	0.27±0.04	*ns*
EU-Nec1	509±21	−24%	c	1.00±0.07	*ns*	0.18±0.03	*ns*
EU-Att1 + EU-Chl1	683±22	+ 1%	a	0.90±0.08	*ns*	0.31±0.05	*ns*
EU-Att1 + EU-Nec1	662±20	−2%	ab	0.89±0.08	*ns*	0.22±0.04	*ns*
PE-Att1 + EU-Chl1	655±23	−3%	ab	0.80±0.07	*ns*	0.23±0.04	*ns*
PE-Att1 + EU-Nec1	661±23	−2%	ab	1.04±0.08	*ns*	0.27±0.04	*ns*

[1] Displayed yields and numbers of fruits are averaged over grafted and non-grafted plants

[2] Superscript numbers indicate treatments that are different at the $p<0.05$ level from other treatments

the correlation with other symptoms, such as yellow spots ($\rho=-0.447$, $p<0.005$) and mosaic ($\rho=-0.335$, $p<0.05$). Both leaf deformation and leaf necrosis affect the total leaf area of the plants.

Discussion

In this study, we examined whether tomato plants can be protected against the detrimental effects of a PepMV infection by a preceding infection with attenuated isolates of this virus. Reviews on cross-protection have identified several disease characteristics that are required before considering this strategy (Fulton 1986; Pennazio et al. 2001). The most important ones are failure to control the disease by eradication, rapid spread of the disease, and losses that are large enough to make reduction by cross-protection the preferred alternative over its potential drawbacks. Finally, the availability of mild virus variants that protect effectively without causing undue harm is a key factor.

PepMV management: eradication and spread

The options to deal with PepMV are currently limited. Growers use certified PepMV-free tomato seeds and can apply hygiene measures that limit viral spread

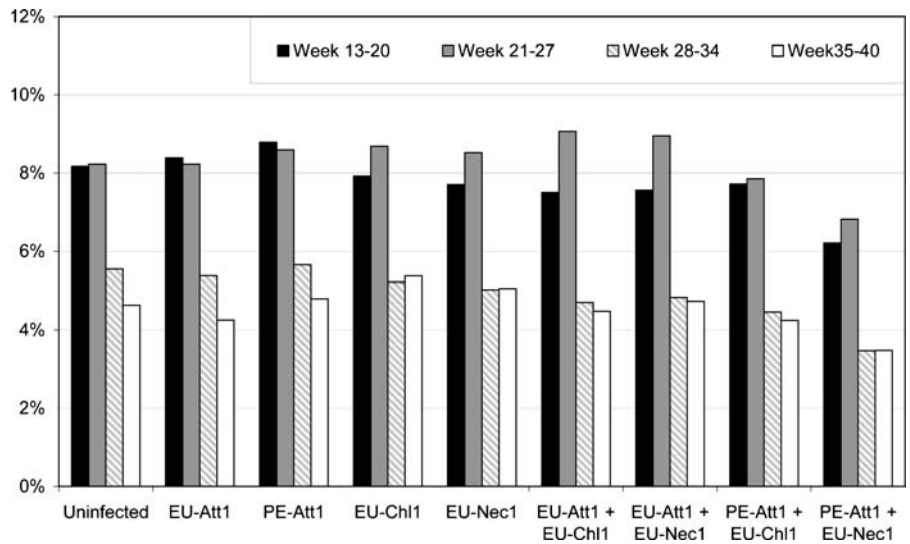

Fig. 4 Percentage of fruits affected by blotchy ripening in uninfected plants and plants infected with PepMV. Percentages were averaged over grafted and non-grafted plants and separated according to the week of harvest

between and within greenhouses. These measures did not prevent PepMV from becoming widespread throughout Europe and North-America (Jorda et al. 2001; Cotillon et al. 2002; Verhoeven et al. 2003; Ling et al. 2008). Symptoms were initially reported as mild (Van der Vlugt et al. 2000) and mature infected plants can appear almost symptomless, which may have contributed to the large scale establishment of PepMV. Development of tomato cultivars with resistance to PepMV might serve as a long term solution. Partial resistance has been identified in the wild tomato species *S. chilense*, *S. peruvianum* and *S. habrochaites* (Ling and Scott 2007; Soler-Aleixandre et al. 2007). Whether these species possess resistance to all PepMV strains is unknown. Although these results are promising, it may take years before resistant cultivars become commercially available.

The extent to which PepMV causes economic losses is still under debate and reported losses vary between studies (Jorda et al. 2001; Spence et al. 2006; Van der Vlugt and Stijger 2008). Differences in symptom severity between isolates of the virus may partly explain this discrepancy. For example, the two attenuated field isolates used in this study caused minimal yield and quality losses, while the two other field isolates caused yield losses of 8 and 24%, respectively. Soler et al. (2000) already reported losses of 20–40%. PepMV is reported to affect fruit quality due to flaming, marbling, blotchy ripening, and fruit size reduction (Spence et al. 2006). Blotchy ripening is potentially related to a PepMV infection and was observed in our study. Its occurrence did, however, not differ between infected and uninfected plants, thus one may question whether the observed "symptoms" are caused by physiological factors or by the pathogen. Other studies have also observed PepMV isolates that do not cause fruit symptoms (Hanssen et al. 2009), and we were not able to obtain EU-strain challenge isolates that consistently caused such symptoms.

Symptom attenuation and effectiveness of cross-protection

The attenuated isolates induced minimal leaf symptoms, had relatively low virus titers, and did not affect yield or fruit quality. Both attenuated isolates were field isolates and, as such, had already demonstrated the ability to survive and to cause systemic infection under greenhouse conditions. The attenuated isolates reduced the effects of PepMV isolates with aggressive symptoms effectively. After a challenge inoculation, total virus accumulation, symptom severity and yield losses were significantly reduced in cross-protected plants compared to non-protected plants. The yields of the cross-protected plants were on a similar level as those of uninfected plants. The most severe leaf symptoms were observed shortly after inoculation regardless of the isolate used for infection, a pattern which has also been observed in other trials (Spence et al. 2006). The challenge inoculations were artificially introduced on all plants simultaneously. In real practice, an infection would involve lower amounts of virus and would spread gradually throughout the greenhouse. As such, the impact of the challenge inoculations represents a worst case scenario and the beneficial effects of protection could, thus, be less than observed in this study.

Overall, symptom severity correlates to PepMV accumulation, but accumulation alone does not explain all differences in symptom severity. For example, *EU-Chl1* and *EU-Nec1* had similar accumulation levels, while the symptoms of *EU-Nec1* were much severer compared to *EU-Chl1*. In turn, symptom severity was negatively correlated to yield. The two symptoms that had the largest effect on yield i.e. leaf deformation and leaf necrosis, affected the leaf area of the plants, which would explain the observed yield losses. The mechanism underlying cross-protection has, so far, remained obscure. In the case of *Chinese yam necrotic mosaic virus* (CYNMV) and *Watermelon mosaic virus* (WMV) attenuated strains prevent the infections of virulent strains from becoming systemic (Kosaka and Fukunishi 1997; Kondo et al. 2007). This would, however, not explain the observed symptom attenuation of PepMV, because the challenge isolates do become systemic in cross-protected tomato plants. The incomplete cross-protection may be due to the incomplete RNA silencing activity (Ratcliff et al. 1999; Valkonen et al. 2002) induced by the protective isolate and thus resulted in the establishment of the aggressive isolates.

Drawbacks of control by cross-protection

A first issue regarding application of cross-protection is the range of PepMV variants against which the attenuated isolates are effective. In this study, we

tested the effectiveness in a "worst case" scenario with two isolates that each cause very severe symptoms. Numerous other virus variants are present in the field and the degree of genetic diversity among isolates is therefore an important factor to consider. Cross-protection is suggested to perform well when the genetic relatedness between protecting and challenging variants is high (Hall et al. 2001; Valkonen et al. 2002). PepMV isolates are grouped into four strains based on sequence similarity. Three of the tested isolates belong to the EU strain of PepMV, while *PE-Att1* belongs to the PE strain. The EU strain predominated in the Netherlands at the time of the trials (Van der Vlugt et al., in prep) as it did in Spain in 2005 and in North America in 2008 (Pagan et al. 2006; Ling et al. 2008). Homogeneity among EU isolates in Europe is high (>99%) (Mumford and Metcalfe 2001; Verhoeven et al. 2003), while the PE strain is highly similar to the EU strain (~94%). This already suggests that cross-protection will function between isolates of both genotypes as was demonstrated in our experiments by the effect of *PE-Att1* on *EU-Chl1* and *EU-Nec1*. Since other EU and PE isolates are within the same range of similarity, cross-protection is expected to function in these cases as well.

Whether cross-protection by *EU-Att1* and *PE-Att1* also functions when they are challenged with isolates of the US1 or CH2 strain is unknown. Sequence similarity between the CH2, US1 en EU/PE strains is approximately 80%, depending on the genomic region (Ling 2007). The US1 strain has not been reported outside North-America until its discovery on the Canary Islands in 2007 (Alfaro-Fernandez et al. 2008). The CH2 strain has already been reported in several European countries around 2005 (Pagan et al. 2006; Hanssen et al. 2007). The CH2 strain was first discovered in the Netherlands in 2005 as well and appears to have increased in prevalence since then (Van der Vlugt et al., in prep). Given the relatively low sequence similarity between the CH2 strain and the EU/PE strains, this recent appearance is concerning with regard to the potential effectiveness of a cross-protection strategy. Moreover, application of cross-protection under these circumstances may result in the emergence of virus variants with new features by recombination between attenuated and challenge isolates. Hanssen et al. (2007) already identified recombinants between the CH2 and the EU strains. To our knowledge, no attenuated CH2 isolates have, so far, been identified.

The host range of PepMV and synergistic effects with other pathogens are two other reasons for caution. PepMV's host range includes mainly *Solanaceae* species and this group encompasses several crop species of economic importance. PepMV systemically infects several cultivars of potato and egg plant (Salomone and Roggero 2002). To what extent the attenuated isolates affect these crops needs to be evaluated as a large-scale introduction of such isolates should not harm productivity or quality of other crops. Large-scale introduction of the attenuated isolates may condition tomato crops for synergistic effects with other tomato viruses. Several reports exist on synergistic interactions between two unrelated viruses in the same plant (Pruss et al. 1997; Karyeija et al. 2000). Gómez et al. (Gómez et al. 2009) found that co-infections of the EU strain and the CH2 strain expanded the range of susceptible hosts. This suggests that when such co-infections occur, because plants that are protected by an EU/PE isolate are challenged by CH2 isolates, this may have unwanted side-effects.

Conclusion

At this moment, application of strict hygiene measures is the preferred management strategy with regard to PepMV. Even within areas with a high infection pressure, it is occasionally possible to remain free of virus. However, problems with PepMV have been increasing since its first occurrence in tomato. The application of cross-protection may represent an attractive method of controlling PepMV in the future. Cross-protection reduces the effects of PepMV isolates that induce severe foliage symptoms and yield reduction, while the effects of the attenuated isolates themselves on yield and fruit symptoms were minimal.

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