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Seed transmission of Pepino mosaic virus and efficacy of the tomato seed disinfection treatments.

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ABSTRACT

Rates of transmitting *Pepino mosaic virus* (PepMV) from seed to seedlings were estimated in seedlings grown from infected tomato (*Lycopersicum esculentum* Mill.) seed. Seed was obtained from symptomatic tomato fruits of plants naturally infected with the virus. To estimate the proportion of seeds infected with PepMV, grouped seeds were assayed for PepMV by ELISA and it turned out to be at least 25%. The seeds were planted and seedlings at the cotyledon and transplant stage were assayed for PepMV by ELISA. Three of 168 seedlings grown from infected seed were PepMV-positive, corresponding to a seed-to-seedling transmission rate of 1.84%. Tomato seed treatments for 24, 48 and 96 hours at 80, 74 and 70ºC respectively, or immersion in 10% trisodium phosphate during 3 hours, or 24 hours in 3g/l pectinase, 3g/l pectinase+2% HCl, 3g/l pectinase+2% HCl+30% commercial bleach, were evaluated. PepMV was largely eradicated with trisodium phosphate. Although treatments at 80 and 74ºC eliminated PepMV in seedlings, the virus was not

eradicated in whole seeds by the treatment. Seed germination was not adversely affected by trisodium phosphate, or the 80 and 74 ºC treatments. The trisodium phosphate treatment can be used to eradicate PepMV in tomato seed without hindering germination.

INTRODUCTION

Pepino mosaic virus (PepMV) was first reported and characterized in *Solanum muricatum* from Peru in 1980 (11). For many years it was thought that the presence of PepMV was restricted to Peru and infected *Solanum muricatum* but no other solanaceous crops grown in this region, such as tomato, potato or eggplant (15). In 1999, PepMV was reported in greenhouse and field-grown tomatoes in The Netherlands (27). The symptomatology is very complex as it depends on the virus isolate, tomato cultivar, temperature and light intensity. Symptoms described include a yellow mosaic of varying intensity on the leaves or single yellow spots between veins, green mosaic, leaf bubbling and other leaf malformations that can be confused with symptoms after the improper use of growth regulators or with CMV infection. However, the most harmful symptom to growers is the yellowish or greenish spots that appear on ripe fruits after harvest, due to an abnormal lycopene distribution or uneven maturation, causing a considerable reduction in market value.

The PepMV introduction in Europe is still not clear, although since the 1999 growing season, the virus spread has rapidly happened throughout the principal production zones in Germany (17), Belgium, United Kingdom (21), Spain (13), Italy (22), USA, Canada (7), France (3) and Austria (28).—see R2 for suggested revision. Mechanical PepMV transmission is easy and effective. Contact between healthy and infected plants as a result of routine handling during cultivation of the crop, is enough to transmit the virus (16) and constitutes the main cause for transmission within a plot. On the other hand, the mechanism for long-distance dissemination of this virus could be assigned to the transfer of infected young plants from the nursery to the grower,

and the use of contaminated seeds (reference missing). Seed transmission of PepMV has been demonstrated in growth chamber trials, although seed transmission has not been proven under field conditions (reference missing).

Seed transmission of PepMV involves two processes (19). First, fruits are infected on seed parent plants. When infected seeds are planted and germinate to produce seedlings they are liable to become infected if PepMV is transmitted from infected fruits to seedlings (i.e., seed-to-seedling transmission). The risk of introducing PepMV on seed to areas where it does not occur is affected by both processes and their influencing factors. This suggests that infected seeds might be a source of primary inoculum for PepMV infections and, combined with routine international distribution of tomato seed, might account for the recent detection of this pathogen in Morocco, Finland, Sweden, Slovakia, Bulgaria, Norway, Denmark, Ukrania, Poland, Hungary, Chile (6), Ecuador (25). Therefore, PepMV is a concern to the tomato industry because of the risk of introducing the pathogen into fields with contaminated tomato seed, resulting in the infection of subsequent crops susceptible to PepMV.

On March 2, 2004, the European Committee published a decree to take measures to control the entry and circulation within the EU of tomato seeds infected with PepMV though inspection and analysis of seeds proceeding from third countries, with the aim of detecting the presence of the virus before allowing their distribution within the EU. Member states are therefore obliged to carry out official inspections for the presence of PepMV in nurseries and seed production stations. An ELISA-based seed health test can be used to estimate relatively low levels of PepMV-infected seeds. If the proportion of infected seed is estimated accurately from seed health tests, the risk of introducing PepMV can be assessed once accurate rates of seed-to-seedling transmission are known.

Different chemical and physical treatments have been reported to eradicate or significantly reduce the incidence of a number of viruses, without adversely affecting seed quality when carried out using precise treatment parameters. Although some seed companies currently utilize these kinds of pre-treatments for tomato seed, the details of these seed treatment protocols are proprietary. Therefore, the objectives of this study were to determine (i) estimates of PepMV seed-to-seedlings transmission rates in order to assess the risk of introducing PepMV in tomato seed more accurately, and to better understand the possible role of PepMV seed transmission in viral perpetuation, perennation, and dissemination, (ii) the efficacy of different chemical and physical seed treatments for the eradication of PepMV from tomato seed and (iii) the effect of these treatments on tomato seed germination .

MATERIALS AND METHODS

The tomato seeds used in this work were from various varieties, originating from tomato plants that were naturally infected under field conditions and identified by the presence of typical PepMV symptoms (ref). These are referred to as 'infected seeds'. Seed from different varieties was not separated, because the infected seeds were provided by Horticultural Cooperatives from several regions in Spain (what regions, and what varieties are dominant in those regions). Two seed batches were used; one harvested in 2001 (lot-01) and another from 2004 (lot-04). Seed was cleaned by rinsing in water followed drying at room temperature on clean muslin.

PepMV seedlings infection test.

To determine seed transmission rates to tomato seedlings, 500 infected seeds were sown into sterile 24-cell trays containing sterilized soil (1:1 turf?? Soil ≠ turf:sand), with a single seed per cell. Trays were incubated in a growth chamber at 25:23ºC (day:night) with a 16:8 h (L:D) photoperiod (Light intensity) and a relative humidity of 60%. Stringent sanitary measures, including isolation, confinement, handling, and insect control, were used to ensure that no spurious virus spread occurred. Fourteen days after sowing, one cotyledon from each of tour plants in a plant-row was pooled into a sample (ca. 0.15 g), homogenized in 3 ml of sample extraction buffer (xx mM SALT? phosphate-buffer, pH xx containing yy% w/v Tween-20). The homogenates were decanted (were they centrifuged?), and 100-µl aliquots were used in an enzyme-linked immunosorbent assay (ELISA), using the commercial antisera against PepMV (DSMZ, Braunschweig, Germany) according to the manufacturer's instructions. Extracts from PepMV-infected and healthy tomato leaves, and buffer alone served as controls. All samples were run in duplicate. Samples were considered positive for PepMV the absorbance at 405 nm was more than twice the mean of the healthy control samples. This screening procedure was repeated when the seedlings reached the four-leaf stage. In this case, a portion of the youngest emerged leaf was harvested from each plant in a row. The four leaves sampled per row were pooled and pulverized with a pestle in 20 volumes of extraction buffer into a plastic bag. To detect PepMV in whole seeds, samples of four infected seed were soaked for 90 min in 1 ml of sample extraction buffer, then ground with a pestle before testing by ELISA.

Estimates of PepMV infection and PepMV transmission rate.

The percentage of seeds or seedlings infected with PepMV was estimated from samples of untreated seed from both seed lots (lot-01 and lot-04) by ELISA. A total of 455 whole seeds assayed

(115 from lot-01 and 340 from lot-04). A total of 260 seedlings at four-leaf stage were assayed (92 from lot-01 seed and 168 from lot-04seed). The seedling infection rate was estimated by the method of Gibbs & Gower (8) using the forumula $p=1-(1-y/n)^{1/k}$, where p= the probability of transmission by a single PepMV-infected seed, y=the number of positive samples, n= the total number of samples assayed, and k=4 (the number of seedlings per sample). A 95% confidence interval was calculated for each estimate (how?).

Seed disinfection treatments.

To determine the efficacy of seed disinfection treatments for eradication of PepMV from tomato seed, two replications were made of the following seven treatments, one replication per seed lot: (i) seeds were heated for 24 h at 80ºC (T80); (ii) seeds were heated for 48 h at 74ºC (T74); (iii) seeds were heated for 96 h at 70ºC (T70); (iv) seeds were submerged in a solution of 10% trisodium phosphate for 3 h, rinsed with distilled water and air-dried on cheese cloth (Q1); (v) seeds were submerged in a solution of 3 g/l pectinase in distilled water during 24 h and washed twice in sterile distilled water (Q2); (vi) seeds were submerged in a solution of 3 g/l pectinase supplemented with 2% HCl in distilled water for 24 h and washed twice in sterile distilled water (Q3); (vii) seeds were submerged in a solution of 3 g/l pectinase supplemented with 2% HCl and 30% of commercial bleach in distilled water for 24 h and washed twice in sterile distilled water (Q4). Approximately 200 seeds were used for the replication of each treatment. For the control group (no treatment) for each replication, 200 seeds were triple-rinsed in sterile deionized water and dried. Prior to use, the treated seed was stored in sterile petri plates sealed with Parafilm.

To detect PepMV infection in treated seeds, half of the seed from each treated group was sown without germination steps?? I don't understand, as described above, and the other half was assayed as whole seeds by ELISA, using the same procedure as with the untreated whole seeds.

142

143 **Data analyses**

144 Analysis of variance, comparison of means using Fisher's protected least significance
145 difference (LSD; $P < 0.05$), correlation coefficients and X^2 test were using STATGRAPHICS Plus
146 ver. 5.1 (publisher?). Possible interactions among factors were identified using ??.

147

148 **RESULTS**

149 No obvious symptoms were observed in any of the tomato seed that tested positive for
150 PepMV by ELISA. As the results obtained with lot-01 were confirmed when the experiment was
151 repeated with lot-04, we only show here the results of the second study. The results of the
152 serological analysis of whole seeds are given in table 1. Eighty-five groups of 4 seeds gave positive
153 results for the ELISA whole seed assay. If only one seed of each group of 4 seeds were infected, this
154 would give a seed infection rate of at least 25% in the seeds of the lot (table 1). A total of 217 seeds
155 from lot-04 were planted, of which 168 produced seedlings. Only 3 samples, corresponding to 12
156 seedlings (4 plants per sample) gave positive in the ELISA analysis. This corresponds to a
157 probability of 0.018 that only one seedling will become infected with PepMV (table 2).

158 The absorbance data from the ELISA test of the untreated seeds of lots 01 and 04 were
159 subjected to analysis of variance of the "absorbance" variable in relation to the factor "stage at
160 which the analysis was performed". From the p-values obtained in the ANOVA (P=0.0000) it can be
161 concluded that the factor "stage of analysis" has a statistically significant effect on the Absorbance
162 of the untreated samples analyzed, with a 95% confidence level. For the analysis of whole seed
163 samples the expected Absorbance value is significantly different to that of the analysis of cotyledon
164 or seedlings. The mean absorbance of untreated seeds (0.252±0.07) is 28 times higher than the mean

absorbance obtained at the transplant stage of seedlings from untreated seeds (0.009±0.007). Performing the serological analyses in the cotyledon or transplant stages gives the same results, i.e. there were no significant differences between the analysis of seedlings at the cotyledon stage and at the transplant stage (figure 1). For this reason the cotyledon analysis data were not used in the subsequent statistical analysis of the data.

Efficacy of Disinfection seed treatments.

All pectinase treatments provoked seed germination during the period of treatment. Therefore it constrained seeding immediately after treatment. Moreover, any seedling from seed treated in the pectinase+HCl+bleach solution emerged after seeding (Table 2). The percentages of seed infection after subjecting the seeds to each of the disinfection treatments are given in table 1, and the rates of PepMV transmission of the seeds treated with each of the different treatments to the seedlings analyzed in each case are given in table 2. While in the seeds treated with T80, T74 and Q2 the infection remained in at least 25% of the seeds, at the seedling stage no infected seedling was found in these treatments. The T70 treatment achieved a reduction of the infection at the whole seed stage, between 23 and 92% of the seeds became infected, but the probability of detecting 1 infected seedling in this treatment remained at 0.016 (tables 1 and 2). An ANOVA of the variable "Absorbance" in relation to the factors "disinfection treatments" and "stage at which the analysis was performed" was carried out. In this variance analysis the absorbance values obtained from the whole seed stage and transplant seedling stage analyses were used from all the treated groups except the seed groups subjected to Treatment Q4, which were eliminated from the statistical analysis, since none of the seeds sown from this treatment germinated. The absorbance values of untreated groups were included as negative controls for the assay. As can be seen in table 3, the simple effects of "analysis" and "treatment", as well as the interaction between both factors, were clearly

significant. The graph of confidence intervals for the effect of "stage" indicated that absorbance was significantly higher for the analyses performed on the seed than those of the four-leaf stage (figure 2). Fisher's procedure of least significant differences (LSD) was applied in the multiple comparisons to determine which means were significantly different from the others. The LSD intervals graph to study the simple effect of "treatment" shows the existence of 5 homogeneous treatment groups with significant absorbance differences among them: Q1-Q3, T80, T70, T74-Q2 and Q2-NT (figure 3). The mean absorbance is much higher in treatments T74, Q2 and NT than in the others, and is also significantly higher in T80 and T20 than in Q1 and Q3. Regarding the analysis of the interaction observed, the difference between the seed and seedling analyses is only significant for treatments T80, T74, T70, Q2 and NT, but not for Q1 and Q3. While the mean absorbance values for the whole seed stage analysis vary within the wide interval of the maximum NT value of 0.252 ± 0.0046 and minimum of 0.0045 ± 0.008 obtained for the Q1 treatment, the mean absorbance values at the transplant stage stay within a narrow interval of values from the maximum obtained with treatment T80, mean value 0.0129 ± 0.0076, to the minimum obtained with Q1, 0.0056 ± 0.0086. All seed treatments significantly reduced the incidence of PepMV in seedlings that developed from treated seeds when compared with seeds that were washed and dried (table 1, table 2).

Effects of the seed treatments on germination of tomato seed

The chi-square test with 7 d.f. and a 95% confidence level demonstrated the existence of significant differences among the percentage of germination obtained with the different treatments. One of the ways of determining any harmful effects of the different disinfection treatments on the seeds is by their influence on germination. The rate of variation in the germination of the seeds treated with different physical and chemical treatments was calculated and compared to the

germination obtained from untreated tomato seeds, for both seed lots. Figure 4 shows the germination results obtained for each treatment and seed lot and the variation observed in this characteristic for each treatment in relation to the untreated group. From the p-values obtained in the ANOVA, it can be concluded that the factor "treatment" has a statistically significant effect on "difference in germination of the seeds" (P=0.0001) with a confidence level of 95%, while the "lot" factor is not significant (P=0.0714). Although, as can be seen in figure 4, the germination obtained from lot-04 was higher in all treatments than that obtained from the lot-01 seeds. The LSD interval graph to study the simple effect of the seed "lot" supports the non existence of significant differences between lots (figure 5). The LSD interval graph in figure 6 shows the simple effect of "treatment" on the variation of germination of treated seeds compared to untreated. The existence of three homogeneous disinfection treatment groups can be distinguished: Q4, T70-Q3-T80-T74-Q2 and T80-T74-Q2-Q1. The difference in germination is significantly higher when seeds were treated with Q4 than with the other treatments. Apart from this, the only pairs of treatments between which significant differences exist are T70-Q1 and Q1-Q3. Treatment Q4 induces a mean reduction in germination of seeds of 68.69±4.1% compared to the mean germination of untreated seeds. Treatment T70 causes a mean reduction in germination of 4.9±4.1%, while Q3 is the treatment that causes the least reduction in germination, with 2.06±4.1%. The other treatments facilitated germination in the seeds from both lots. Treatment T80 showed the lowest increase in germination, with only 4.46± 4.1%, followed by T74 with an increase of 7.13± 4.1%. Q2 was within the same range with an increase in germination of 7.37± 4.1%, and finally, the treatment that showed the highest increase in germination in comparison with the untreated seed lots was Q1 with a 13.16± 4.1% increase in germination of treated seeds. From the analysis of figure 6 it can be determined that, with a confidence level of 95%, there are significant differences between treatments Q1-T70

and Q1-Q3 and between treatment Q4 and the other disinfection treatments used, regarding their effect on the percentage germination of the seeds of both treated lots.

DISCUSSION

This is the first report on seed transmission of PepMV. Previous reports indicated that certain PepMV isolates could not be seed transmitted in tomato cv. Camone (24). The small size of the sample tested in the previous study, 52 seedlings, might account for the very low transmission rate in the host tested. These authors raised the possibility of seed transmission among other tomato cultivars and other *Lycopersicon* species used for grafting *Lycopersicum esculentum*. It is not uncommon to find extremes of seed transmission rates when tested in different plant species or cultivars (5; 29). We detected PepMV in at least 25% of the tested tomato seeds, but only 1.84% of the progeny derived from the same seed lot were infected. Our data confirm that the incidence of viral transmission by seed does not necessarily correlate with the rate of infected progeny seedling (10; 20)—make it clear that this does not relate to PepMV. The rate of seed transmission is not necessarily a good indicator of epidemiological significance. Low rates of seed transmission, in conjunction with secondary spread by insect vectors or mechanical transmission can result in the introduction of viruses into new areas and can produce viral disease epidemics (4). Lettuce production is severely affected by lettuce mosaic potyvirus (LMV) when a 0,001% incidence of seed transmission occurs, because of subsequent spread by aphid vectors (23). Similar low rates of seed transmission are also sufficient for the development of epidemics of BYMV in subterranean clover pastures (12). Thus, even extremely low rates of seed transmission can facilitate the introduction of viruses into new crop production areas (10).

Our tests with PepMV demonstrated very low levels of seed transmission. PepMV is an extremely stable and persistent virus, with relatively high rates of unintentional? mechanical

transmission, quite similar to Tobacco mosaic virus (TMV). TMV remains viable when carried in contaminants on the seed surface, and seedling infection occurs primarily by mechanical transmission, especially during handling of the seedlings (1; 26). We are convinced that PepMV is transmitted via seeds, as contaminant and debris that stick to the seeds, on the seed surface—what data convinces you?. Presumably, tomato seeds acquire PepMV particles during their development on the infected plant. We do not know the where PepMV is located in the seed, but the elimination of this virus from seed lots by trisodium phosphate treatment suggests the virus is predominantly carried on the seed surface. It is possible that the germinating seedling gets inoculated with virus located on the seed coat as it emerges from the seed This is similar to TMV transmission from infected tomato seeds, where the embryo is not infected and the virus is transmitted to seedlings from the seed coat through small wounds (18). This mechanism might also account for the low rates of PepMV seed transmission, even though the level of seed infection is quite high. Moreover, it is possible that PepMV incidence could increase if seedlings were to be transplanted—this statement doesn't really follow.

From this study we conclude that PepMV can be introduced into tomato production areas through seed transmission. It is possible that the virus was introduced into Europe through infected seed, and under certain environmental conditions PepMV can spread rapidly from very few primary infection sites due to its facility for transmission by contact (16). The ideal control of PepMV would be to prevent the introduction of the causal agent into the field. Infested seed represents one of the mechanisms through which the virus may be introduced into a tomato field. Other means of introduction would be infected transplants, contaminated implements, and possibly, infected wild plants (2; 14). PepMV has been shown to be seed-transmitted in seeds harvested from fruit with the typical marbling symptom. It is necessary to eliminate fruits and seedlings between the rows that came from the germination of the seeds of the infected fruits that fall to the ground. There is no way

283 to detect and cull the fruit that are symptomless carriers of PepMV. Therefore, we would
284 recommend not harvesting seeds from fruit in the vicinity of symptomatic fruit. The safest approach
285 would be to harvest seeds only from fields that had no PepMV infection. Since it is difficult to be
286 absolutely sure that harvested seed is not contaminated with PepMV, a treatment to eradicate the
287 pathogen from the seeds at harvest is another approach to obtaining noninfested seed. Trisodium
288 phosphate pre-treatment has been used to eliminate tobacco mosaic virus (TMV) from infected
289 tomato seeds. It does not affect seed germination rates and leads to healthy tomato plants (9).
290 Immersion of the seed infected with PepMV, in trisodium phosphate solution was effective in
291 eliminating seed transmission as Na_3PO_4 eliminated assayable PepMV from the whole seed.
292 However, the virus was not eradicated from all of the seeds submited to the thermics treatments (80,
293 74 and 70ºC) neither with the treatment with only pectinase. There did not seem to be any difference
294 in the effectiveness of 80ºC, 74ºC or only pectinase treatments under our conditions. Emergence
295 was sometimes reduced by 70ºC and the two complex treatments of pectinase. Treatment at 70ºC for
296 96 h was relatively ineffective. The virus was not eradicated from all seeds, there was still a very
297 low level of seed transmission (1.6%) and germination was affected. In contrast, we found pectinase
298 to be as effective as trisodium phosphate, with no infected seedlings and with a positive effect on
299 germination rate. Trisodium phosphate followed by Q2, T80 and T74 treatments was most effective
300 in eliminating PepMV contamination of tomato seed. Neither of these chemical treatments adversely
301 affected seedling emergence, instead of this germination was higher than in the untreated control.
302 Using precisely controlled parameters, trisodium phosphate can be used to eradicate PepMV in
303 tomato seed without hindering germination. Moreover, trisodium phosphate treatment is the easiest
304 and the most rapid of all the treatments assayed, as only 3 h of treatment are sufficient for the
305 elimination of PepMV in whole seed. Therefore this is the treatment that should be recommended.
306

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