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Transmission of *Pepino mosaic virus* by fungal vector *Olpidium virulentus*

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ABSTRACT

Transmission of *Pepino mosaic virus* (PepMV) by fungal vector *Olpidium virulentus* was studied in two experiments. Two characterized cultures of the fungus were used as stock cultures for the assay: culture A from lettuce roots collected in Castellón (Spain), and culture B from tomato roots collected in Murcia (Spain). These fungal cultures were maintained in their original host and irrigated with sterile water. The drainage water collected from irrigating these stock cultures was used for watering PepMV-infected and non-infected tomato plants to constitute the acquisition-source plants of the assay, which were divided into six different plots: plants containing fungal culture A (non-infected and PepMV-infected); plants containing fungal culture B (non-infected and PepMV-infected); PepMV infected plants without the fungus; and plants non-infected either with PepMV and the fungus. Thirty-six healthy plants grouped into six plots, which constituted the virus acquisition-transmission plants of the assay, were irrigated with different drainage waters obtained by watering the different plots of the acquisition-source plants. PepMV was only transmitted to plants irrigated with the drainage water collected from PepMV-infected plants whose roots contained the fungal culture B from tomato with a transmission rate of 8%. No infection was detected in plants irrigated with the drainage water collected from plots with only a fungus or virus infection. Both the virus and fungus were detected in water samples collected from the drainage water of the acquisition-source plants of the assay. These transmission assays demonstrated the possibility of PepMV transmission vectored by *O. virulentus* collected from tomato crops under the study conditions.

KEYWORDS: PepMV, virus transmission, tomato, *Potexvirus*

INTRODUCTION

Pepino mosaic virus (PepMV) was first reported in pepino (*Solanum muricatum* Ait.) in Peru (Jones et al., 1980). In 1999, this virus was detected in protected tomato crops (*Solanum lycopersicum* L.) in the Netherlands (Van der Vlugt et al., 2000). Nowadays, this virus spreads through the main tomato production areas of Europe and America (EPPO, 2008). PepMV is a member of the genus *Potexvirus*, which has filamentous particles with a normal length of 508 nm (Jones et al., 1980). This virus produces variable symptoms that depend on the virus isolate, tomato cultivar, temperature and light intensity. Typical leaf symptoms generally include yellow or light-green mosaic, interveinal single yellow spots, and bubbling or other leaf distortions such as filiform. Fruits typically show marbling, an alteration of the fruit colour, resulting in uneven ripening which considerably lowers their market value (Jordá et al., 2000).

PepMV is readily transmitted mechanically and spreads between plants easily by contaminated tools, hands, clothing, and by direct plant-to-plant contact (Wright & Mumford, 1999). Furthermore, bumble bees, such as *Bombus terrestris* L., *B. canariensis* Pérez and *B. impatiens* (Cresson), which are commonly used as pollinators in tomato greenhouses, have also been implicated to experimentally spread PepMV between tomato plants through the direct injuring of flowers or through fertilization with infected pollen (Lacasa et al., 2003; Shipp et al., 2008). On the other hand, the long-distance dissemination mechanism of this virus may occur through the transfer of young infected plants from the nursery to the grower, through infected grafts, cuttings or fruits, and even through the recently demonstrated seed-to-seedling transmission (Córdoba et al., 2007). PepMV transmission has been studied in a recirculating hydroponic system where the transmission rate was quite high and its spreading could

remained unnoticeable given the lack of symptoms in the infected tomato plants (Fakhro et al., 2005, Schwarz et al., 2007).

Simultaneously with the first outbreak of PepMV infection in Spain (Jordá et al., 2001a), a new syndrome associated with the presence of the virus occurred in Murcia which was referred to as 'tomato collapse' because of the wilting appearance of the affected plants. The first symptom was slight reversible wilting which occurred at midday to later become irreversible, and the plant died. This syndrome was reproduced under controlled conditions in different assays which demonstrated that PepMV, together with the fungal vector *Olpidium brassicae* (Wor.) Dang senso lato (sl), were involved in the 'tomato collapse' syndrome (Córdoba et al., 2004b). Other authors suggested that 'tomato collapse' would be associated with necrosis of the vascular system caused by PepMV accumulation; however collapsed symptoms were not reproduced in that study and third Koch´s postulate was not observed (Soler-Aleixandre et al., 2005).

O. brassicae sl, a member of the Chytridiales order, is a root-infecting parasite fungus involved in several plant virus transmissions, for example, *Lettuce big-vein virus* (LBVV, *Varicosavirus*) (Campbell, 1996). This fungus presents three developmental stages during its life cycle: zoospores, zoosporangia and resting spores. The fungus survives from crop to crop as resting spores that produce zoospores. A zoospore encysts on the epidermal cells of host roots. The thallus becomes embedded in the host cytoplasm and later develops into either a thin-walled zoosporangium or a thick-walled stellate resting spore. When mature, zoosporangia release zoospores through exit tubers and the life cycle is repeated (Campbel, 1996; Temmink & Campbell, 1968).

Host specialisation is an important characteristic of *O. brassicae* sl, and different strains or isolates of the fungus have been widely described, such as the crucifer strain,

which requires zoospore mating to develop resting spores, or the non-crucifer strain, which does not require sexual mating for resting spores formation (Campbell & Sim, 1994; Koganezawa et al., 2005). Given these differences and the molecular analysis of the complete rDNA-ITS regions of the fungus, the crucifer and non-crucifer strains of *O. brassicae* sl are considered different species. A new species named *Olpidium virulentus* (Sahtiyanci) Karling was proposed for the non-crucifer strain of the fungal vector (Koganezawa et al., 2005), which was later confirmed by Sasaya and Koganezawa (2006). These new approaches for the *Olpidium* species nomenclature will be used in this study.

The confirmed relationship of *O. virulentus* and PepMV in the syndrome referred to as `tomato collapse`, the ability of *O. virulentus* to infect different plants species and to transmit several plant viruses, together with the need for detailed research into putative ways of PepMV transmission, led to this study being developed. The main objective of the present work was to evaluate the possibility of PepMV transmission to healthy tomato plants by fungal vector *O. virulentus*.

MATERIALS AND METHODS

Fungal vector cultures

Two different cultures of the fungal vector *O. virulentus* were used in the assay: Culture A and Culture B, which were previously characterised molecularly by Herrera-Vásquez *et al.* (2009). These cultures consisted in soil samples collected from lettuce crops (Culture A), showing typical symptoms of LBVV, and from tomato crops (Culture B), with collapse symptoms, where the fungus had been previously detected. No other micro-organisms were present in these soil samples. The characteristics of fungal vector cultures A and B are provided in Table 1. Routine maintenance of stock

culture B and vector transmission experiments were performed with tomato plants (cv. Marmande) grown under stringent sanitary conditions and planted in a three-times sterilised (120ºC for 30 min) mixture of sand and peat (1:3) substrate. Plants were grown in 30 cm-diameter plastic pots and were kept in growth chambers at 26ºC/22ºC (day/night) with a 12-h photoperiod and 60% relative humidity. Stock culture A was routinely maintained in lettuce plants to keep the fungus in the roots of its original host. To avoid possible contamination with other *Olpidium* spp. isolates, all the plants were watered with sterile water during the assay. Pots which contained fungal vector cultures were placed 5 cm over a tray to collect the drainage water used in the transmission assay and to avoid further contaminations. All the precautions taken were to control *Olpidium* spp. and to prevent accidental spread into uninoculated root systems.

Virus isolates

Three PepMV isolates were used in this work and their characteristics are shown in Table 1. The two 3672 and 4809 isolates were previously studied molecularly in three different zones of the PepMV genome: partial RNA-dependent RNA polymerase gene (RdRp), triple gene block gene (TGB), and coat protein gene, (CP), as in Pagán *et al.* (2006), and they were characterised within the European PepMV strain in all the zones studied. The third isolate, 4988, was characterised in this work as previously described (Pagan et al., 2006). The sequence information obtained from the three zones of the genome was submitted to the GenBank database (accession numbers are provided in Table 1). The transmission assay was replicated twice in August-November 2005 (Expt. 1) and in November-March 2006-07 (Expt. 2). During Expt.1, all three PepMV isolates were studied. In Expt. 2 however, only isolate 4988 was analysed given the results previously obtained in Expt. 1.

Preparation of the acquisition-source plants (P_0)

Tomato cv. Marmande seeds, disinfected with 10% trisodium phosphate for 3h as described by Córdoba *et al.* (2007), were sown in well trays filled with a three-times sterilised (120ºC for 30 minutes) mixture of sand and peat (1:3). At the four leaf stage, plants were transplanted into 30 cm-diameter plastic pots. Until that time, plants were watered with sterile water and kept according to stringent isolation measures to avoid other *Olpidium* spp. contaminations.

Plants were distributed into 3 plots according to the drainage water applied to them, as described in Fig. 1. Plot A_0 was constituted by 6 plants irrigated with the drainage water obtained from culture A of *O. virulentus*. Plot B_0 was constituted by 6 plants irrigated with the drainage water obtained from culture B of *O. virulentus.* Plot H_0 was constituted by 6 plants irrigated with sterile water. *O. virulentus* inoculative irrigation of the plants of plots A_0 and B_0 commenced immediately after transplanting. This inoculative irrigation consisted in irrigation with the zoospore suspensions (ranging from $1x10^5$ to $1x10^6$ zoospores per mL, estimated by the method of Campbell (1988)) contained in the drainage water obtained from irrigating the stock-pots cultures A and B of *O. virulentus* with 5L of sterile water. Those stock-pots and plants which constituted plot H_0, were always irrigated with sterile water. P_0 plants were maintained in a growth chamber under controlled temperatures (26ºC/22ºC day/night), and inoculative irrigation (approximately 500mL of drainage water collected from stock-pots A or B) was performed once a week. These irrigations were complemented with other non-inoculative irrigations with sterile water depending on the plants necessities.

Three plants within each plot were inoculated with PepMV to constitute sub-plots AP_0 (adquisition-source plants irrigated with culture A and inoculated with a PepMV isolate), BP_0 (adquisition-source plants irrigated with culture B and inoculated

with a PepMV isolate), and HP_0 (adquisition-source plants irrigated with sterile water and inoculated with a PepMV isolate). The rest of the plants in each plot were maintained without PepMV inoculation, thus constituting sub-plots An_0 (adquisition-source plants irrigated with culture A and non-inoculated with any PepMV isolate), Bn_0 (adquisition-source plants irrigated with culture B and non-inoculated with any PepMV isolate), and Hn_0 (adquisition-source plants irrigated with sterile water and non-inoculated with any PepMV isolate).

In Expt. 1, the three PepMV isolates (3672, 4809, 4988; Table 1) were inoculated to one plant of sub-pots AP_0, BP_0 and HP_0, as shown in Fig. 1. In Expt. 2 however, only the 4988 isolate was inoculated to the three plants of these sub-plots. Fig. 1 represents the design of Expt. 1. The inoculation of the plants belonging to sub-plots AP_0, BP_0 and HP_0 was performed with an inoculum prepared by grinding leaf material from PepMV-infected plants in inoculation buffer (0.01M phosphate buffer pH 7.2 containing 0.2% sodium bisulphite and 0.2% sodium diethyldithiocarbamate DIECA) in 1:4 (wt/v), using carborundum (600 mesh) as an abrasive.

In order to confirm correct PepMV inoculation in plots AP_0, BP_0 and HP_0, and to ensure non-infection of plots An_0, Bn_0, Hn_0 in both stock cultures A and B, all the plants (P_0) were analysed 15 dpi (days post-inoculation) against PepMV by the double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) using a specific antiserum supplied by DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH. Braunschweig, Germany) following the manufacturer's instructions. One month after the first inoculative irrigation, presence of the fungus in P_0 roots was verified by observing the samples collected from the secondary roots of the plants P_0, which were previously clarified by following the method described by Jordá *et al*. (2002) using a Nikon-YS-100 light microscope (Nikon Corporation, Tokyo,

Japan). Fungal infection was monitored by quantifying the number of resting spores (rs) and zoosporangia (zs) in these secondary roots samples. Three 3 cm-long slices of secondary roots per plant were randomly visualised and quantified on a 0-3 scale (0= any fungal structure present; 1 = range 0-100 fungal structures, rs or zs; 2 = range 101-1000 fungal structure, rs or zs; 3 = more than 1001 fungal structures, rs or zs). P_0 plants constituted the acquisition-source plants for both the virus and the fungal vector.

PepMV detection in the drainage water of acquisition-source plants (P_0)

The drainage water obtained from irrigating the different sub-plots (AP_0, An_0, BP_0, Bn_0, HP_0, Hn_0) of P_0 was processed in both Expt. as illustrated in Fig. 2 to check the presence of both the virus and the vector in water samples. P_0 were irrigated with 500 mL of the corresponding irrigation source, as explained before, at 45 d.p.i. (days post inoculation). A volume of 200 mL of drainage water from each sub-plot (AP_0, An_0, BP_0, Bn_0, HP_0, Hn_0) was collected and filtered through musslin. The filtrate was centrifuged at 13000 rpm, 4ºC for 2h in a Sorvall® DuPont Company centrifuge with a RC-5B GSA rotor (Delaware, USA). Two different fractions were obtained with this centrifugation: a pellet (part A) and a supernatant (part B), which were processed separately as shown in Fig. 2.

Part A consisted in one obtained pellet which was resuspended in 5 mL of Milli-Q sterile water and centrifuged at 30000 rpm for 2h 30 min in a Beckman Optima™ L-90K ultracentrifuge with an SW-41 rotor (Fullerton, California). The pellet was resuspended in 1 mL of Milli-Q sterile water. The total RNA extraction procedure with the RNAwiz extraction kit (Ambion, Hungdinton, United Kingdom) was performed with 500 µL of the obtained suspension following the manufacturer's instructions. The total DNA of the rest of the suspension (500 µL) was extracted with the EZNA Plant

DNA Miniprep kit (Omega, Biotech, Doraville, USA) following the manufacturer's instructions. In order to detect PepMV in the RNA extracted from the resuspended pellet, reverse transcription-polymerase chain reaction (RT-PCR) was performed using the SuperScript II one step RT-PCR system with the Platinum Taq DNA polymerase kit (Invitrogen Life Technologies. Barcelona, Spain) with specific primers which amplify the complete coat protein gene (CP) of the virus (Pagán et al., 2006). On the other hand, a multiplex PCR (polymerase chain reaction) assay for the simultaneous detection and differentiation of *Olpidium* spp. was performed, as described before, according to Herrera-Vásquez *et al.* (2009) to detect the fungus in the DNA extracted from the resuspended pellet.

The other part of the process, Part B, consisted in a supernatant which was processed by the concentrating virus method for use in water samples described by Gosálvez *et al.* (2003). The drainage waters of AP_0 and BP_0 were processed together, as were those of An_0 and Bn_0, to constitute a drainage water sample of PepMV-inoculated plants containing the fungal vector (AP_0+BP_0), and virus-free plants containing the fungal vector (An_0+Bn_0), respectively. The drainage water of the plants inoculated with PepMV without the fungal vector (HP_0), along with that of healthy plants (Hn_0), were also included in the assay as controls. The RNA extracts obtained were analysed by RT-PCR against PepMV-CP as described before to detect the presence of the virus in the drainage water of P_0.

All the amplified PCR products were analysed on 1.2% agarose/TAE gels stained with ethidium bromide. The products obtained were compared with a DNA standard marker (GeneRuler^{TM} 100 bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania).

Virus acquisition and transmission by the vector to the acquisition-transmission plants (P_1).

Thirty-six tomato plants, cv. Marmande, were sown and grown in seedbeds until transplanted to 20 cm-diameter plastic pots, as described before. These plants were to constitute the acquisition and transmission plants of the assay (P_1). P_1 plants were classified into six different plots depending on the inoculation source that they would receive (Fig. 1). P_1 plots were AP_1 (constituted by the 6 acquisition-transmission plants irrigated with the drainage water obtained from AP_0), An_1 (constituted by the 6 acquisition-transmission plants irrigated with the drainage water obtained from An_0), BP_1 (constituted by 6 the acquisition-transmission plants irrigated with the drainage water obtained from BP_0), Bn_1 (constituted by the 6 acquisition-transmission plants irrigated with the drainage water obtained from Bn_0), HP_1 (constituted by the 6 acquisition-transmission plants irrigated with the drainage water obtained from HP_0) and Hn_1 (constituted by the 6 acquisition-transmission plants irrigated with the drainage water obtained from Hn_0). Inoculative irrigation started immediately after transplanting and was performed at the same frequency as described before for P_0. Each inoculative irrigation consisted in the drainage water obtained from P_0 which had been filtered through Watman No. 4 filter paper to eliminate plant debris from the solution in order to avoid the possible mechanical transmission of the virus. P_1 plants were irrigated with equal volumes (250 mL) of filtered drainage water obtained from irrigating P_0.

Serological analysis of leaf samples by DAS-ELISA was carried out for all the P_1 plants, as described before, at 45 days after beginning the inoculative irrigation. The analysis was repeated with all the P_1 plants every 15 days until the end of the assay. The total RNA of P_1 tomato leaves were extracted and analysed by RT-PCR with

PepMV-CP specific primers as described before to confirm the results obtained by the serological analysis.

Observation and monitoring the fungus in the secondary roots by light microscope was performed with all the P_1 in both Expt. 1 and 2, 60 days after transplant and at the beginning of the inoculative irrigation, as described before. In Expt. 2 however, root samples were also molecularly analysed by multiplex PCR, after extracting the total DNAs from 0.05g of root tissues, as detailed before.

Intensive and thorough precautions were taken in all the steps of this study to control *Olpidium,* to prevent accidental spread into uninoculated root systems, and to avoid PepMV plant-to-plant transmission through contact.

RESULTS

Molecular characterisation of the viral isolates

Isolate 4988 (GenBank Accession numbers detailed in Table 1) corresponded to the European tomato strain (EU) of PepMV, as the already characterised isolates 4809 and 3672 did (Pagan et al., 2006), which in the BLAST analysis showed a high nucleotide identity with percentages of 99% between them and the EU strain isolates published in the GenBank database (GenBank Accession numbers AJ438767, AJ606360).

Previous analysis performed to the acquisition-source plants (P_0)

In the serological analysis against PepMV performed at 15 dpi to the leaves of P_0, 100% of the inoculated plants with the virus were positive (sub-plots AP_0, BP_0 and HP_0) and negative in all the plants of the rest of the sub-plots (An_0, Bn_0 and Hn_0) (Table 2), resulting in successful PepMV inoculation. In Expt. 1, the symptoms observed in the

PepMV-inoculated plants depended on the isolate. Isolates 3672 and 4809 were very mild, plants inoculated with isolate 3672 only developed a slight bubbling on the youngest leaves, and isolate 4809 was asymptomatic in all the inoculated plants. However, those plants inoculated with isolate 4988 developed more aggressive symptoms such as dark green mosaic, bubbling and nettle heads in both Expt. 1 and Expt 2. For that reason, only isolate 4988 was used in Expt. 2 for the transmission assay.

A morphological observation of P_0 roots confirmed the presence of *O. virulentus* given the presence of stellate resting spores (Fig. 4a) in 100% of the plants belonging to plots AP_0, An_0, BP_0 and Bn_0 and which had been irrigated with the stock cultures of *O. virulentus* A and B (Table 2). Other fungal structures were also observed in these plants, such as zoosporangia (Fig. 4a). During Expt. 1, the quantification of the fungal structures was similar in all the plots containing fungal structures. However in Expt. 2, the plots irrigated with the stock culture B of *O. virulentus* generally presented more infection, as revealed the fungal structures (rs and zs) quantification which was codified mainly as 2 (ranged from 101-1000), than those plots irrigated with culture A, which fungal structures (rs and zs) were quantified as 1, ranged 0-100 (Table 2).

PepMV detection in the drainage water of acquisition-source plants (P_0)

The total RNA obtained from Part A, used to process drainage water, was seen to be positive via RT-PCR with the specific primers for the CP gene of PepMV (expected amplicon of 845 bp) in the processed drainage water of plots AP_0 and BP_0, which had been inoculated with the both fungus and the virus in Expt. 1; however only BP_0 was positive in Expt. 2 (Fig. 3a).

The results of the multiplex PCR analysis for the differentiation of *Olpidium* spp., performed with the DNA extraction obtained in Part A, was positive for *O. virulentus* (expected amplicons of 579 bp) in all the processed drainage water of plots P_0 which had been irrigated with the fungal cultures (AP_0, An_0, BP_0 and Bn_0), as shown in Fig. 3b.

The RT-PCR assay conducted with the specific primers for the CP gene of PepMV performed with the total RNA extracted from Part B was seen to be positive only in the mixture of drainage water of AP_0+BP_0. The rest of the extracted samples consisting in the drainage water of virus-free plants containing the fungal vector (An_0+Bn_0), the drainage water of plants inoculated with PepMV without the fungus (HP_0), and healthy plants (Hn_0) were negative according to the RT-PCR assay against PepMV (Fig. 3c).

Virus acquisition and transmission by the vector to the acquisition-transmission plants (P_1).

In Expt.1, one plant of plot BP_1 showed the typical dark green mosaic and bubbling symptoms on the leaves associated with PepMV infection one month after starting inoculative irrigation with the drainage water of the P_0 plants (Fig. 4b). DAS-ELISA performed with all the P_1 plants proved positive only in this symptomatic plant. RT-PCR analysis further confirmed this ELISA result. This symptom was similar to those which developed in the P_0 plants inoculated with isolate 4988. In Expt. 2, no typical symptoms of PepMV were observed during the assay. DAS-ELISA performed one month after starting irrigation with the drainage water of the P_0 plants was negative for all the P_1 plants. However, the RT-PCR assay proved positive for one plant of plot BP_1 (Table 4). Therefore, the transmission rate of both assays was 8%, and this

percentage was calculated as one positive plant of twelve plants which could have been infected (plots AP_1 and BP_1) by PepMV transmission vectored by *O. virulentus*.

The observation of the roots in both experiments revealed the presence of stellate resting spores which are characteristic of *O. brassicae* sl (Fig. 4a), and zoosporangia were noted in the P_1 plants irrigated with the drainage water of plots An_0, AP_0, Bn_0 and BP_0. No fungal structure was observed in the P_1 plants irrigated drainage water of plots Hn_0 or HP_0, which were fungus-free plants (Table 4). The quantification of the fungal structures in Expt. 1 revealed that the P_1 plants, which had been irrigated with culture B of *O. virulentus*, presented more quantities of fungal structures than those irrigated with culture A, which also occurred in the acquisition-source plants, P_0. In Expt. 2, no differences in the quantification of fungal structures were observed among plots irrigated with culture A or B (Table 4).

In Expt. 2, the results of the multiplex PCR assay to differentiate *Olpidium* ssp. performed with the roots of P_1 coincided with the morphological observation of the roots; only those plants irrigated with the drainage water containing fungal cultures (An_1, AP_1, Bn_1 and BP_1) tested positive for *O. virulentus*.

The density of the root system of all the plants irrigated with and without the fungus was compared "*de visu*" at the end of the assay, and a clear difference was observed; those plants irrigated with any of the *O. virulentus* cultures presented a high reduction (15-35%) of root system density than fungus-free plants.

DISCUSSION

PepMV, as a member of the genus *Potexvirus*, easily and quickly spreads through tomato crops, causing important economic losses. Some forms of PepMV transmission associated with its mechanical transmission, such as transmission by

Bombus spp., which carried the virions stuck to their bodies and their contact with plants produced the transmission of the virus (Lacasa et al., 2003), or through infected pollen (Shipp et al., 2008), have been studied. Recently, PepMV seed transmission has been proved (Córdoba et al., 2007) and constitutes a perennation mechanism as well as the long-distance transport of the virus. Some common weed species were also reported to be PepMV infected, which constituted a possible virus reservoir (Jordá et al., 2001b; Córdoba et al., 2004a). Therefore, these facts represent a high risk of PepMV distribution given its easily mechanical transmission. The present study expands the information about the epidemiology of this virus, where PepMV was vector-transmitted into two different replicate assays by the fungal vector *O. virulentus* with a transmission rate of 8%. Another *Potexvirus*, *Potato virus X* (PVX), was reported to be vector transmitted by *Synchitrium endobioticum* (Schilb.) Perc. under experimental conditions; however this form of transmission was not corroborated in later investigations (Šutić et al., 1999).

Throughout this study, apart from the plants infected with both the fungus and virus (AP_0, BP_0 in the assays), all the controls or checks as described by Campbell (1988) were included; fungus alone (An_0, Bn_0 in the assays) to verify that zoospores were produced in virus-free conditions; virus alone (HP_0 in the assays) to check that there was no mechanical transmission or vector contamination; and the usual non-inoculated plants to test that experimental plants were produced and were maintained free of both the virus and the vector (Hn_0 in the assays). These controls were never contaminated during both experiments (Expt.1 and Expt. 2). Moreover, only those plants irrigated with drainage water containing both the virus and fungal vector succeeded in transmitting PepMV.

Vector-free drainage water obtained from irrigating the plants infected with PepMV (HP_0) did not transmit the virus to healthy plants P_1. This ensures that PepMV was not transmitted in the irrigation water alone without the presence of the vector. This result contrasts with some studies which suggest that PepMV is distributed through a recirculating hydroponic system (Fakhro et al., 2005; Schwartz et al., 2006). In some cases, PepMV infection was delayed because of the pre-infection of *Phytium aphanidermatum* (Edson) Fitzp. (Schwartz et al., 2006). However, the presence of *Olpidium* spp. zoospores in either the recirculating solution or the tomato roots was not checked in those works. In Almería (Spain), *Olpidium bornovanus* (Sahtiyanci) Karling, an efficient vector of *Melon necrotic spot virus* (MNSV), was found in irrigation pools destined to water cucurbit crops, constituted a possible source of MNSV contamination and spread (Gómez & Velasco, 1991). The possibility of *O. virulentus* contamination in the irrigation systems has to be taken into account to avoid possible PepMV transmission which may occur, as this study has evidenced. Although the transmission rate of PepMV by *O. virulentus* was low in this study compared with other viruses vectored by this fungus which presented variable transmission rates, e.g., *Mirafiori lettuce virus* (MiLV) and *Lettuce big vein virus* (LBBV) with 95% (Lot et al., 2002), 40% of *Lettuce ring necrosis virus* (LRNV) (Campbell & Lot, 1996) or 30-40% of MNSV (Tomlinson & Thomas, 1986), PepMV has the extraordinary capability of mechanical spread in tomato crops. The low transmission rate obtained in this study could be a result of the method employed in this assay, which differed from that described by Campbell (1988) who inoculated a zoospore suspension of the fungus mixed with purified virions. In the present study, the intention was to reproduce the real situation in fields where intensive and reiterated cultures of tomato with recirculating irrigation systems are commonly used. Therefore, the plant which presented PepMV

vector transmission was irrigated with the drainage water from plants infected with both the virus and the fungus.

In addition, interaction between PepMV and *O. brassicae* sl has been demonstrated in the syndrome referred to as `tomato collapse` (Córdoba et al., 2004b). Therefore, not only the potential risk of PepMV transmission by the fungus, but also the manifestation of an aggressive syndrome that causes wilting in tomato crops, meant that suitable control measures had to be taken to avoid fungal spread. *O. brassicae* sl survived in soil as resting spores for 20-22 years, but zoospores and vegetative sporangia were killed by short drying periods (Campbell, 1985). Despite the thermal death point of resting spores of *O. brassicae* s.l. being reported to be near 65ºC for 10 minutes (Campbell & Lin, 1976), the fungus was able to survive in infected soil after composting when high temperatures (50-70ºC) were reached (Bollen et al., 1989). Therefore, all these characteristics have to be considered when controlling fungal spread. Disinfection treatments of the irrigation solution with surfactant agral (alkalyn phenol ethylene oxide) (Tomlinson & Thomas, 1986) and ultraviolet treatment (Campbell, 1996) are two examples of effective measures to control *Olpidium* infections.

Moreover, a method described by Gosalvez *et al.* (2003) for the detection of MNSV in water samples was performed with samples of the drainage water obtained from irrigating the adquisition-source plants (P_0) to detect PepMV in water samples. The virus was detected in the drainage waters obtained from plants that presented both the virus and the fungi (AP_0, BP_0 in the assays). In the first centrifugation (13000 rpm for 120´), all the fungal structures were contained in the pellet because just 1000g (3000-4000 rpm) for 10´ is enough to concentrate *O. bornovanus* zoospores (Tomlinson & Thomas, 1986). The result of the presence of *O. virulentus* in the pellet obtained from

the drainage water was expected. Nonetheless, the virus was only detected in the pellets from the drainage waters obtained from P_0 plants which contained the fungus. Yet the presence of the fungus was needed to detect the virus in the pellet, and even the supernatant was analysed to verify the presence of PepMV in the water tested as only positive in the sample which corresponded to the drainage water from P_0 plants containing both the fungus and the virus. Therefore, the virus itself was not detected in the drainage water, and could not be transmitted to the acquisition-transmission plants (P_1) without the presence of *O. virulentus*.

The transmission of PepMV only occurred in this study with the fungal vector collected from tomato roots, and never with the culture originating from lettuce roots. The host specificity of *O. brassicae* s.l. (even the new renamed species *O. virulentus*) and *O. bornovanus*, which measures reproductive fitness or compatibility between a fungus isolate and a host plant, is one of the widely demonstrated characteristics of these fungal vectors (Campbell & Sim, 1994; Campbell et al., 1995; Koganezawa et al., 2005). The ability to transmit a given virus may be limited to certain host-specific strains of the vector. A high specificity of virus adquisition by the zoospore membranes of the fungus is required for transmission by fungal vectors (Campbell, 1996). Likewise, an inefficient vector requires either more virus or more zoospores, specific hosts, or the combination of all these items for transmission, which occurred with the melon and cucumber strains of *O. bornovanus* that transmitted *Squash necrosis virus* (SqNV) to watermelon, but not to other compatible hosts (Campbell et al., 1995). The inability of zoospores to adsorb virus particles correlated with the failure of the zoospores of some *O. brassicae* strains to transmit *Tobacco necrosis virus* (TNV). Perhaps these zoospores have fewer or none specific sites of adsorption and, therefore, seemed to adsorb fewer particles of some viruses (Temmink et al., 1970). In this report, the viral transmission

was also observed to be restricted to a tomato isolate of *O. virulentus*, although the lettuce isolate, reproduced properly in tomato roots as the quantification results indicated given the plurivorous nature of this species. This is an approach for the evaluation of the specificity between fungus vector and virus, but further studies are required to verify such specificity.

On the other hand, the three PepMV isolates used in this study belonged to the European tomato strain. PepMV presents a high molecular variability and different strains of the virus have been described. The results presented herein are preliminary, and other assays are being performed to check the ability of other strains of the virus to be vector-transmitted by *O. virulentus*.

Furthermore, *Olpidium* spp. have always been considered fungal vectors that cause conspicuous damage to the roots of the host. However, reduced root density between those plants inoculated and non-inoculated by the fungus was evident, as reported by Campbell & Sim (1994) who observed the browning of roots and reduced development in the stock culture plants inoculated with *O. brassicae*. A reduction in root and shoot growth of 20-50%, and increased respiration of roots, have been attributed to an unidentified *Olpidium* spp. in melons (suspected to be *O. bornovanus*) (Hadar et al., 1992). Similarly, the root systems of infected and non-infected roots resulted in clear developmental differences in the present study.

The importance of constantly inspecting plants at the seedling stage to avoid the early spread of mechanical transmissions of PepMV and to control the presence of the fungal vector in water or soil, which could represent a potential risk for PepMV transmission in hydroponic systems, or even in soil cultures, can never be underestimated or ignored.

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TABLES

Table 1: Characteristics of the *O. virulentus* cultures and PepMV isolates used in the assay.

O. virulentus						**PepMV**					
Culture	**Origin**	**Original host**	**Collection date**	**Microscopic observation[a]**	**Molecular characterisation**	**Isolate**	**Collection date**	**Origin**	**Tomato cultivar**	**Symptoms[b]**	**Molecular characterisation**
A	Castellón,Spain	Lettuce	2001	rs, zs	EU981901[c]	**3672**	2000	Murcia	Gabriela	W, FM	AM042588[d] AM041933[d] AM313791[d]
B	Murcia, Spain	Tomato	2001	rs, zs	EU981902[c]	**4809**	2001	Murcia	nd	W	AM042568[d] AM041934[d] AM113792[d]
						4988	2001	Las Palmas	Daniela	YM, W	FJ384784[e] FJ384786[e] FJ384785[e]

[a] rs: stellate resting spores, zs: zoosporangia.
[b] FM: Fruit Marbling, W: Wilt, YM: yellow mosaic.
[c] *O. virulentus* isolated molecularly characterized by Herrera-Vásquez *et al*. (2008). Accession numbers published in the GenBank database.
[d] PepMV isolates molecularly characterised by Pagán *et al.* (2006). Accession numbers published in the GenBank database
[e] Nucleotide sequences obtained in this work as described by Pagán *et al*. (2006) and submitted to the GenBank database.
nd: unknown

Table 2: Serological analysis of the leaves against PepMV and monitoring of *Olpidium* spp. by microscopic observation of the roots performed with the stock fungal cultures and the acquisition-source plants (P_0).

		Expt.1				Expt.2			
	Irrigation source[a]	**PepMV isolate inoculated**	**Plot[b]**	**As-PepMV**	**Monitoring *Olpidium* spp.[c]**	**PepMV isolate inoculated**	**Plot[b]**	**As-PepMV**	**Monitoring *Olpidium* spp.[c]**
Culture A	**H**	none	Culture A	-	rs=2, zs=2	none	Culture A	-	rs=2, zs=2
Culture B	**H**	none	Culture B	-	rs=2, zs=2	none	Culture B	-	rs=2, zs=2
P_0[b]	**A**	3672		+	rs=2, zs=2			+	rs=1, zs=1
		4809	AP_0	+	rs=2, zs=2	4988	AP_0	+	rs=1, zs=1
		4988		+	rs=2, zs=2			+	rs=1, zs=1
		none		-	rs=2, zs=2	none		-	rs=1, zs=1
		none	An_0	-	rs=2, zs=2	none	An_0	-	rs=1, zs=1
		none		-	rs=2, zs=2	none		-	rs=1, zs=1
	B	3672		+	rs=2, zs=3			+	rs=2, zs=3
		4809	BP_0	+	rs=2, zs=2	4988	BP_0	+	rs=2, zs=2
		4988		+	rs=2, zs=2			+	rs=1, zs=1
		none		-	rs=2, zs=2	none		-	rs=2, zs=2
		none	Bn_0	-	rs=2, zs=2	none	Bn_0	-	rs=2, zs=2
		none		-	rs=2, zs=2	none		-	rs=1, zs=1
	H	3672		+	rs=0, zs=0			+	rs=0, zs=0
		4809	HP_0	+	rs=0, zs=0	4988	HP_0	+	rs=0, zs=0
		4988		+	rs=0, zs=0			+	rs=0, zs=0
		none	Hn_0	-	rs=0, zs=0	none	Hn_0	-	rs=0, zs=0

[a]Irrigation performed with three different sources: A= drainage water obtained from the irrigation of fungal culture A; B= drainage water obtained from the irrigation of fungal culture B; H= sterile water.

[b]Plants P_0 (acquisition-source plants) which were grouped into different plots: Culture A= stock culture of *O. virulentus* collected from lettuce crop soil; Culture B= stock culture of *O. virulentus* collected from tomato crop soil; AP_0= acquisition-source plants irrigated with culture A and inoculated with PepMV isolates; An_0= acquisition-source plants irrigated with culture A and non-inoculated with any PepMV isolate; BP_0= acquisition-source plants irrigated with culture B and inoculated with PepMV isolates; Bn_0= acquisition-source plants irrigated with culture B and non-inoculated with any PepMV isolate; HP_0= acquisition-source plants irrigated with sterile water and inoculated with PepMV isolates; Hn_0= acquisition-source plants irrigated with sterile water and non-inoculated with any PepMV isolate.

[c]Monitoring of *Olpidium* spp. structures, resting spores (rs) or zoosporangia (zs), present in the roots of the plants by light microscope observation following the scale: no fungal structure = 0 ; range 0-100 rs, zs = 1; range 101-1000 rs, zs = 2 ; more than 1001 rs, zs= 3.

Table 3: Results of the analysis performed with the acquisition-transmission plants (P_1) to detect the possible transmission of PepMV and to confirm the presence of *O. virulentus*.

Plot[a]	Expt. 1					Expt. 2					
	PepMV[b]			*Olpidium* spp.		PepMV[b]			*Olpidium* sp.		
	Symptoms observation	DAS-ELISA	RT-PCR	Microscopic observation		Symptoms observation	DAS-ELISA	RT-PCR	Microscopic observation		PCR[d]
				Presence	Monitoring[c]				Presence	Monitoring[c]	
AP_1	0/6	0/6	0/6	6/6	rs.=2, zs=1	0/6	0/6	0/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
An_1	0/6	0/6	0/6	6/6	rs.=2, zs=1	0/6	0/6	0/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
BP_1	1/6	1/6	1/6	6/6	rs.=3, zs=2	0/6	0/6	1/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
Bn_1	0/6	0/6	0/6	6/6	rs=2, zs=2	0/6	0/6	0/6	6/6	rs=2, zs=2	6/6 (*O.vir*)
HP_1	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6
Hn_1	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6	0/6	0/6	0/6	rs.=0, zs=0	0/6

[a] Plants P_1 (acquisition-transmission plants) which were grouped into different plots: AP_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of AP_0. which were infected with both *O. virulentus* culture A and PepMV; An_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of An_0, which were infected only with *O. virulentus* culture A; BP_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of BP_0, which were infected with both *O. virulentus* culture B and PepMV; Bn_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of Bn_0, which were infected only with *O. virulentus* culture B; HP_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of HP_0, which were infected only with PepMV; Hn_1= acquisition-transmission plants irrigated with the drainage water collected from the irrigation of Hn_0, which were free of *O. virulentus* and PepMV infection.

[b] Number of positive plants/total number of plants analysed.

[c] Monitoring of Olpidium spp. structures, resting spores (rs) or zoosporangia (zs), present in the roots of the plants by light microscopic observation following the scale: no fungal structure = 0 ; range 0-100 rs, zs = 1; range 101-1000 rs, zs = 2 ; more than 1001 rs, zs= 3.

[d] *O.vir*= Resulted in the amplicon that correspond to *O. virulentus* (579 bp).

FIGURE LEGENDS

Figure 1: Schematic representation of the experimental set-up of the transmission assay. The legend of the different images represented is included in the rectangle in the right of the figure.

Figure 2: Basic steps involved in the extraction procedure of PepMV and *O. virulentus* from the drainage water of the acquisition-source plants (P_0).

Figure 3: Molecular analysis of water samples collected from the drainage water of the acquisition-source plants (P_0) processed as described in Fig. 2. **a.** Analysis by one-step RT-PCR using specific primers for PepMV, CP-D and CP-R described by Pagán *et al.* (2006) performed with the total RNA obtained from part A of the method which corresponded to samples collected from the drainage water of plants AP_0 (Lane 1 and 7), An_0 (Lane 2 and 8), BP_0 (Lane 3 and 9), Bn_0 (Lane 4 and 10), HP_0 (Lane 5 and 11) and Hn_0 (Lane 6 and 12), during Expt. 1 (Lanes 1-6) and Expt. 2 (Lanes 7-12). The expected amplicons corresponding to PepMV positive result are indicated. Lanes PC: positive control infected with PepMV, NT: no RNA template, M: 100 bp molecular weight marker. **b.** Multiplex PCR analysis using specific primers for the simultaneous detection of *Olpidium* spp. (Herrera-Vásquez et al., 2009) performed to the total DNA obtained from part A of the method which corresponded to samples collected from the drainage water of plants AP_0 (Lane 5), An_0 (Lane 6), BP_0 (Lane 7), Bn_0 (Lane 8), HP_0 (Lane 9) and Hn_0 (Lane 10). The expected amplicons corresponding to *O. bornovanus*, *O.virulentus* and *O. brassicae* are indicated. Lanes PC: positive control of each *Olpidium* sp. (lane 1: *O. bornovanus*, lane 2: *O. virulentus*, lane 3: *O. brassicae*), NT:

no DNA template, M: 100 bp molecular weight marker. **c.** Analysis by one-step RT-PCR using specific primers for PepMV, CP-D and CP-R described by Pagán *et al*. (2006) performed with the total RNA obtained from part B of the method which corresponded to samples collected from the drainage water of plants AP_0 and BP_0 mixed (Lane 1), An_0 and Bn_0 mixed (Lane 2), HP_0 (Lane 3) and Hn_0 (Lane 4). The expected amplicons corresponding to PepMV positive result are indicated. Lanes PC: positive control infected with PepMV, NT: no RNA template, M: 100 bp molecular weight marker.

Figure 4: **a**. Stellate resting spores characteristic of *O. brassicae* sl, and zoosporangia. **b.** The typical green mosaic and bubbling symptoms on the leaves of one plant from plot BP_1 associated with PepMV infection one month after the beginning of the inoculative irrigation with the drainage water of the P_0 plants during Expt. 1.