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Identification and partial characterisation of *Lettuce big-vein associated virus* and *Mirafiori lettuce big-vein virus* in common weeds found amongst Spanish lettuce crops and their role in lettuce big-vein disease transmission

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Abstract

The potential role of 10 frequently occurring weed species found amongst Spanish lettuce crops as host plants for the two viruses associated with the lettuce big-vein disease, *Lettuce big-vein associated virus* (LBVaV) and *Mirafiori lettuce big-vein virus* (MLBVV), was studied. The results showed that both viruses can infect naturally growing *Sonchus oleraceus* (common sowthistle) plants, the unique susceptible species detected among the analysed weeds. The sequences of the coat protein (CP) genes of the LBVaV and MLBVV isolates recovered from *S. oleraceus* plants were determined. Phylogenetic studies revealed a very close relationship between the CP sequences from these weed isolates and those from Spanish lettuce. Moreover, we showed that *S. oleraceus* can act as a source of lettuce infection by means of *Olpidium brassicae*, the vector fungus of both viruses.

Introduction

Lettuce big-vein disease occurs in all major lettuce-growing areas through the world and becomes a serious problem when nutrient film technique cultures are used. Typical symptoms begin with a chlorotic clearing around the leaf veins followed by leaf distortion, maturity delay and reduction of head size. The affected plants are hence unsuitable for marketing and this frequently forces farmers to ban production. Low temperatures and reduced light intensity increase the severity of symptoms (Walsh, 1994).

Many efforts have been made to identify the causal agent of the disorder since it was first reported by Jagger and Chandler (1934). A viral etiology has been described involving two different viruses: *Mirafiori lettuce big-vein virus* (MLBVV) and *Lettuce big-vein associated virus* (LBVaV). The serological proximity to *Tobacco stunt virus* (Vetten et al., 1987) resulted in the classification of LBVaV into the genus *Varicosavirus*. MLBVV has been included by specific morphology, serology and other characteristics into the genus *Ophiovirus* (Roggero et al., 2000). LBVaV and MLBVV have a segmented genome of ssRNA and virus particles contain RNA molecules of both polarities. Negative-sense RNAs predominate for LBVaV while MLBVV contain nearly equimolar amounts of RNA molecules of both polarities (Sasaya et al., 2001, 2002, 2004; van der Wilk et al., 2002; Kawazu et al., 2003). Recently, it has been

proposed that MLBVV but not LBVaV is the causal agent of lettuce big-vein disease (Lot et al., 2002). However, symptomatic plants serologically negative for MLBVV were reported to be positive for LBVaV in a survey carried out in open fields in Italy (Roggero et al., 2003), very probably due to environmental variability and sensitivity of the detection procedures.

Both viruses are naturally acquired by the chrytrid fungus *Olpidium brassicae in vivo* and then vectored from root to root (Roggero et al., 2003). This fungus–viruses association represents a serious problem in the control of big-vein disease spread in the field since viruses may survive crop to crop within the resting spores (RS) of the fungus that can remain in state for decades (Campbell, 1965, 1996). Moreover, the density of virulent RS can be increased by maintaining continuous production of lettuce in the same area. This situation leads to an early infection of lettuce roots by the fungus that may result in severe outbreaks of the disease (Navarro et al., 2004).

Olpidium brassicae has a wide range of host plants (Campbell, 1965; Temmink et al., 1970; Campbell and Sim, 1994). However, big-vein symptoms have only been observed in the field on lettuce (*Lactuca sativa*) and endive (*Cichorium endivia*) crops and after artificial inoculation of the virulent fungus on spiny sowthistle (*Sonchus asper*), common sowthistle (*Sonchus oleraceus*) and on different species of *Lactuca*, suggesting a narrower range of host plants for the viruses than for the fungus (Campbell, 1965; Jones, 2004). In this scenario, rotating crop host to fungi but not viruses may reduce the amount of virulent fungus present in soils and the severity of the disease.

On the other hand, it is possible that both fungus and viruses share a common host different from commercial crops such as agricultural weed species that may allow the persistence of the disease in periods with no production. The detection of weeds which act as a natural reservoir for the disease is difficult since many different weeds can also become infected with viruses without showing symptoms. The only advice that has been offered until now is to eradicate with no discrimination every plant surrounding the crops. However, the removal of all vegetation would not be possible without economic or environmental consequences. Chemicals spent in weed control and soil erosion could permanently affect the local fauna and flora. A more comprehensive solution to this could be a thorough inspection to identify hazardous hosts by recognizing those weed species where viruses can accumulate. Recently, different LBVaV and MLBVV molecular detection tools have been developed including ELISA, multiplex RT-PCR and molecular hybridisation (Roggero et al., 2003; Navarro et al., 2004). This work deals with the study of the presence of LBVaV and MLBVV on weeds occurring in the close proximity of lettuce crops and the partial molecular characterisation of these weed isolates. The potential capability of two common weeds to act as infection sources via *Olpidium brassicae* was also studied.

Materials and methods

Plant material

Plant samples of several species of agricultural weeds were collected during three consecutive years (2001, 2002 and 2003) and analysed for the presence of both MLBVV and LBVaV by a previously developed dot-blot molecular hybridisation method (Navarro et al., 2004). The selected species included: *S. oleraceus* and *Senecio vulgaris* in the family Compositae; and *Chenopodium murale* and *Chenopodium glaucum* in the family Chenopodiaceae. Other weeds belonging to the families Brassicaceae (*Sisymbriium irio*), Malvaceae (*Lavatera* sp.), Primulaceae (*Anagallis arvensis*), Aizoaceae (*Mesembryanthemum nodiflorum*) and Papaveraceae (*Fumaria pugsleyana* Líden) were also tested. None of the sampled weed plants or those observed in the field showed any type of symptoms. The surveys were performed at the 'grupo PRIMAFLOR' lettuce production areas located in Almería (SE Spain), which showed high big-vein incidence. In addition, in 2003 one of the coldest Spanish production areas located in Granada (SE Spain), was also screened due to the high incidence of big-vein symptoms. The sample number of every plant analysed was related to the abundance of the species in the field at the time of collection, done from within and at the border of the production area. Samples were also taken along roadsides and irrigation channels. Seeds from *S. vulgaris* and *S. oleraceus* were collected in a commercial 'iceberg' lettuce field (SE Spain, Pulpí; Almería). An

'iceberg' variety (*L. sativa* cv. Toro) was used for experiments on infection transmission by *O. brassicae*.

O. brassicae *isolates Virulent origin and maintenance*

Soil with *O. brassicae* isolates transmitting big-vein disease were provided by 'grupo PRIMAFLOR'. In order to eliminate the possibility of different *O. brassicae* isolates being present in the original source of inoculum, a diseased lettuce stock without original soil was established. Iceberg lettuce seeds (cv. Toro) were sown in plastic trays using vermiculite as substrate and inoculated three times with a zoospore suspension of the fungus (5×10^4 zoospores ml^{-1}) to ensure infection. Zoospores were obtained by soaking the roots in tap water of symptomatic plants, which had been grown in the original infected soil. Zoospore presence was microscopically confirmed and their density was calculated with a haemocytometer. All trials were carried out at a constant temperature (18 °C), with a photoperiod of 14 h light/10 h darkness and with 70% relative humidity.

Nucleic acid extraction

Nucleic acids were extracted following a procedure that avoids the use of organic solvents, adapted from the method described by Dellaporta et al. (1983) for viroids (Astruc et al., 1996) and viral RNAs (Sánchez-Navarro et al., 1998, 1999). Briefly, 0.5 g of fresh leaf tissue was manually homogenised in sealed plastic bags in the presence of 2 ml of extraction buffer (100 mM Tris–HCl, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol). Homogenate aliquots (0.5 ml) (excluding plant remnants) were transferred to a 1.5 ml tube and incubated with 25 µl of 20% SDS at 65 °C for 20 min. Then, 125 µl of 5 M potassium acetate was added and the mixture was maintained at 0 °C for 20 min. The tubes were then centrifuged at $8000 \times g$ for 15 min to remove unwanted plant debris. Nucleic acids were recovered from the supernatant by ethanol precipitation, resuspended in 50 µl of sterile water and used directly in dot blot hybridisation experiments. Alternatively, the RNA from one sample for each botanical species was precipitated overnight at 4 °C with LiCl 2 M. The tubes were centrifuged at $8000 \times g$ for 20 min and the pellet was washed with 70% ethanol, dried, and resuspended in 100 µl sterile DEPC treated water. An aliquot of 4 µl of these samples was analysed by electrophoresis in formaldehyde 1.8% agarose gel (Sambrook et al., 1989).

Dot blot hybridisation

The nucleic acid samples were denatured by incubation at 60 °C for 15 min in the presence of $6 \times$ SSC and 7.4% formaldehyde. Each sample (4 µl) was applied directly onto the nylon membranes, air-dried, and cross-linked by exposure to UV irradiation. The membranes were prehybridised for at least 1 h at 68 °C and hybridised overnight at the same temperature with specific LBVaV and MLBVV digoxigenin labelled riboprobes of positive polarity. Both riboprobes were previously reported and correspond to complete sequences of the coat protein (CP) genes of both viruses (Navarro et al., 2004). Chemiluminescent detection with CSPD reagent as substrate was performed as recommended by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). *Taq* DNA Polymerase buffer (Roche Molecular Biochemicals, Mannheim, Germany) was used. After a 15 min incubation at 70 °C, the resulting 3′ A-tailed fragments were ligated into pGEM®-T Easy Vector (Promega Corp., Madison, WI, USA), (Kobs, 1997). Recombinant plasmids were transformed into competent *Escherichia coli* strain DH5α. Nucleotide sequences of the MLBVV and LBVaV fragments inserted in the recombinant plasmids were obtained using an automated sequencer.

Phylogenetic analysis

Clustal X (1.8), a windows interface for the Clustal W multiple sequence alignment programme, was used to obtain the multiple alignments of nucleotide and predicted amino acid sequences of MLBVV and LBVaV CPs (Thompson et al., 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (www.megasoftware.net) (Kumar et al., 2001). Evolutionary distance estimation for nucleotide sequences was measured by the number of amino acid substitutions using the Poisson correction method. Topology of trees was deduced by means

of the minimum evolution distance method with 10,000 bootstrap replicates.

Monitoring O. brassicae *infection*

Fungal infection was microscopically monitored in lettuce roots. Five 1 cm-long slices of roots per plant were randomly taken and stained with Tryptan Blue, with RS and zoosporangia (Z) being visualised by light microscopy. The quantification of the infection involved measuring the mean number of RS and Z per centimeter.

Results

Incidence of LBVaV and MLBVV in agricultural weeds

A total of 346 samples corresponding to nine different species belonging to seven families were analysed for the presence of LBVaV and MLBVV. None of the sampled weed plants or those observed in the field showed any type of symptoms. Representative results for the detection of LBVaV and MLBVV by non-isotopic molecular dot-blot hybridisation (Navarro et al., 2004) in the different weed species analysed are shown in Figure 1. Complete data from the analysis are summarised in Table 1. Both viruses were only detected in *S. oleraceus* plants. However, single LBVaV but not MLBVV infection was observed in six *S. oleraceus* plants. MLBVV was always detected simultaneously with LBVaV as a double infection in four samples.

Diversity and phylogenetic analysis of the CP gene among isolates of MLBVV and LBVaV obtained from S. oleraceus *and lettuce plants*

Sequence information of the LBVaV and MLBVV isolates from infected *S. oleraceus* plants collected in commercial lettuce crops was obtained in order to investigate their relationship with those isolates affecting lettuce plants. The LBVaV and MLBVV CP genes from the doubly infected *S. oleraceus* samples from Almeria, in addition to those from the two LBVaV infected plants from Granada, were cloned and sequenced (Table 2) as described by Navarro et al. (2005). LBVaV and MLBVV isolates obtained from the same plant were named with an identical number. The similarity values among the LBVaV sequences from *S. oleraceus* plants obtained varied between 96–100% at the nucleotide level and 97–100% at the amino acid level. Comparison with the published LBVaV CP sequences from lettuce isolates resulted in similarity values ranging from 94% to 99% for nucleic acid sequences and from 96% to 99% for the deduced amino acid sequences. In the case of

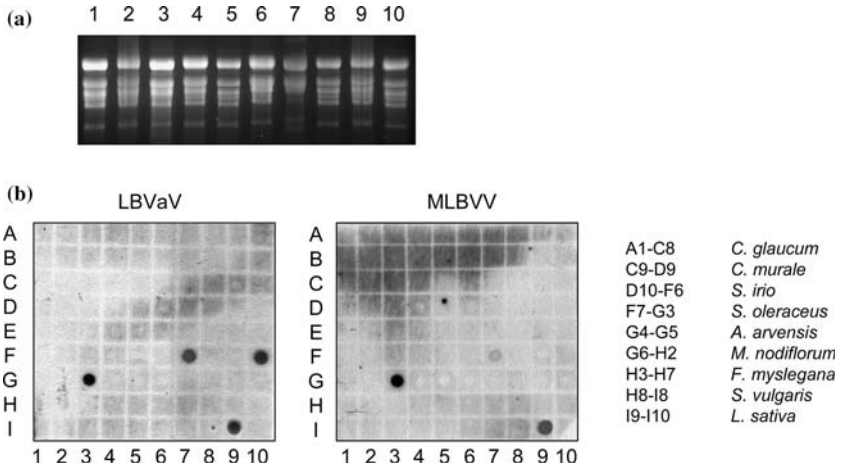

Figure 1. a) Electrophoresis in formaldehyde 1.8% agarose gel of the RNAs from one sample of each botanical species analysed: *S. oleraceus* (1); *S. vulgaris* (2); *C. glaucum* (3); *C. murale* (4); *S. irio* (5); *F. myslegana* (6); *Lavatera* spp. (7); *A. arvensis* (8); *M. nodiflorum* (9) and *L. sativa* (10). b) Representative results from the detection of LBVaV and MLBVV in weeds by non-isotopic molecular dot-blot hybridisation. Analyzed plants correspond to 88 weed samples obtained from a survey performed at Almeria (SE Spain). Species names are indicated on the right. Nucleic acids from double infected (I9) and uninfected (I10) lettuce were used as positive and negative controls, respectively.

Table 1. Detection of LBVaV and MLBVV by dot-blot molecular hybridisation in several weeds collected from two different Spanish regions, Almeria and Granada

Region	Date	Weed species	Family	LBVaV	LBVaV + MLBVV
Almería	2001	*Senecio vulgaris*	Compositae	0/40	0/40
		Sonchus oleraceus	Compositae	1/7	0/7
	2002	*Senecio vulgaris*	Compositae	0/40	0/40
		Sonchus oleraceus	Compositae	4/61	2/61
	2003	*Chenopodium glaucum*	Chenopodiaceae	0/28	0/28
		Chenopodium murale	Chenopodiaceae	0/21	0/21
		Sisymbrium irio	Brassicaceae	0/27	0/27
		Sonchus oleraceus	Compositae	3/7	2/7
		Lavatera spp.	Malvaceae	0/9	0/9
		Anagallis arvensis	Primulaceae	0/2	0/2
		Mesembryanthemum nodiflorum	Aizoaceae	0/7	0/7
		Fumaria pugsleyana	Papaveraceae	0/5	0/5
Granada	2003	*Senecio vulgaris*	Compositae	0/31	0/31
		Sonchus oleraceus	Compositae	0/5	0/5
		Sisymbrium irio	Brassicaceae	0/2	0/2
		Senecio vulgaris	Compositae	0/26	0/26
	2003	*Sonchus oleraceus*	Compositae	2/6	0/6
		Lavatera spp	Malvaceae	0/11	0/11
		Chenopodium murale	Chenopodiaceae	0/11	0/11

MLBVV, the similarity values among weed sequences were between 98–99% at the nucleotide level and 99–100% at the amino acid level. Comparison with the available MLBVV CP sequences from lettuce isolates resulted in similarity values ranging from 87% to 98% at the nucleotide level but from 93% to 99% at the amino acid level.

LBVaV and MLBVV CP nucleic acid sequences from weeds were included in a phylogenetic study together with the CP sequences of geographically distinct isolates of both viruses obtained from lettuce plants and available in the database. The analysis was performed using the minimum evolution method. As a result of the low statistical support values for several interior branches, the phylogenetic relationships among LBVaV or MLBVV isolates are presented as a condensed tree where branches with less than 50% of the bootstrap support were collapsed (Figure 2). Therefore, branch lengths are not proportional to the number of nucleotide substitutions. In the case of the LBVaV analysis, the CP gene of *Sonchus yellow net virus* (SYNV) was selected as an outgroup sequence in order to root the tree (Figure 2). The resulting tree topology and clustering of lettuce isolates was similar to that previously obtained (Navarro et al., 2005). SYNV clearly branches apart from lettuce and *S. oleraceus* LBVaV isolates which form a monophyletic group. Five of the LBVaV sequences from weed isolates fell into a relatively homogeneous group constituted by almost all of the Southeast Spanish isolates. The SON1 sequence was not included in this cluster being related to the GAL1 Northwest Spanish isolate (Figure 2).

Table 2. Name of virus isolates from *S. oleraceus* used for phylogenetic analysis, geographic origin and EMBL accession numbers of the CP gene sequences

Virus	Isolate	Locality	Accession number
LBVaV	SON1	Almeria	AY839618
	SON2	Almeria	AY839619
	SON3	Almeria	AY839620
	SON4	Almeria	AY839621
	SON5	Granada	AY839622
	SON6	Granada	AY839623
MLBVV	SON1	Almeria	AY839624
	SON2	Almeria	AY839625
	SON3	Almeria	AY839626
	SON4	Almeria	AY839627

Phylogenetic relationships among the MLBVV isolates were also examined by the minimum evolution method. The topology of the tree generated with nucleotide sequences showed that the MLBVV isolates from lettuce and *S. oleraceus* plants form a monophyletic group separated from *Citrus psorosis virus* (CPsV) which was included in the analysis as a different ophiovirus or outgroup taxa. Two clearly differentiated phylogenetic groups were observed and denominated as subgroup A and B (Navarro

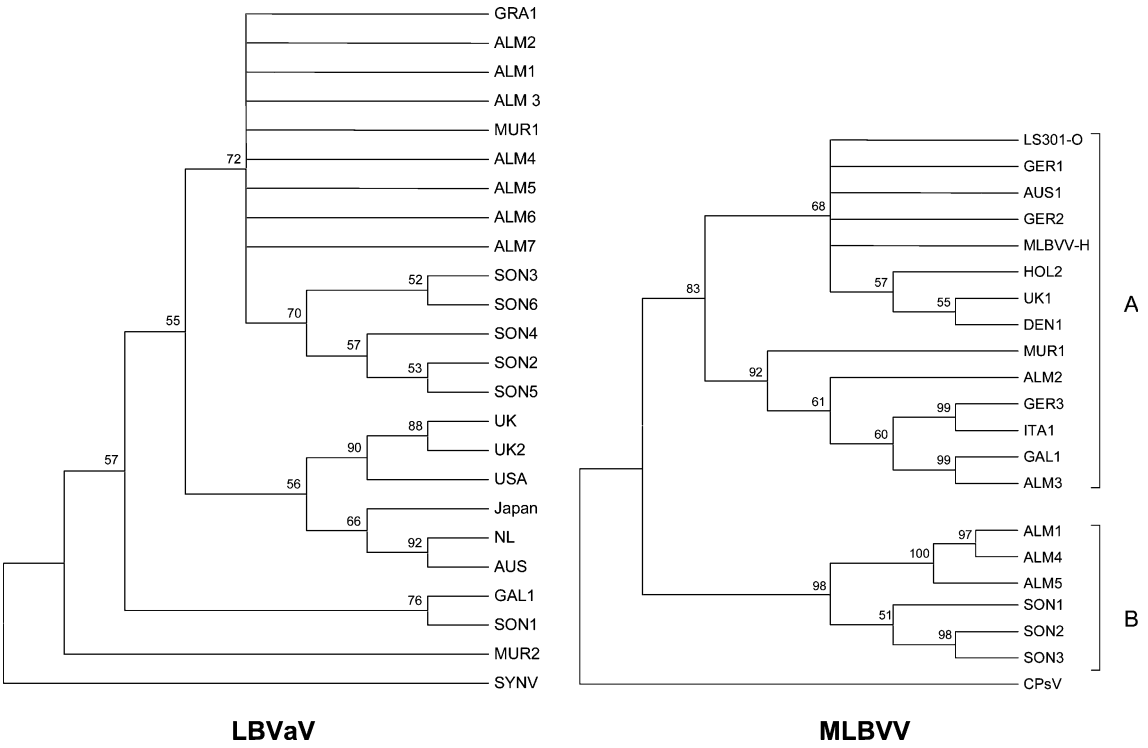

Figure 2. Phylogenetic analysis of LBVaV (left) and MLBVV (right) isolates based on their CP nucleotide sequences. The dendrogram was constructed with the minimum evolution method. The numbers above nodes indicate the percent of bootstrap replicates in which those nodes were recovered. Internal branches with less than 50% bootstrap support were collapsed. For a complete description of the weed isolates see Table 2. The LBVaV and MLBVV trees were rooted with the CP gene of *Sonchus yellow net virus* (SYNV) or *Citrus psorosis virus* (CPsV), respectively. The sequences of each isolate were named using the initials of the country of origin and the virus name; the numbers following the letters indicate different isolates obtained from the same country (Navarro et al., 2005). EMBL accession numbers of the LBVaV sequences used are: MUR1 (AY366411); ALM1 (AY366414); ALM2 (AY366413); ALM3 (AY581684); ALM4 (AY581685); ALM5 (AY581686); ALM6 (AY581687); ALM7 (AY581688); GAL1 (AY366412); GRA1 (AY581689); MUR2 (AY581691); USA (AY496053); NL (AY496056); AUS (AY496055); UK (AY496054); UK2 (AY581690) and Japan (AB050272). EMBL accession numbers of the MLBVV sequences used are: MUR1 (AY366415); ALM1 (AY366417); ALM2 (AY366418); ALM3 (AY581700); ALM4 (AY581701); ALM5 (AY581702); GAL1 (AY366416); HOL2 (AY581693); GER1 (AY581695); GER2 (AY581697); GER3 (AY581698); AUS1 (AY581696); UK1 (AY581694); DEN1 (AY581692); ITA1 (AY581699); MLBVV-H (AF532872) and LS301-O (AF525935).

et al., 2005). Interestingly, all MLBVV weed isolates, together with three Spanish lettuce isolates (ALM1, 4 and 5), clustered in the subgroup B showing 98% and 99% sequence similarity with these isolates at the nucleotide and amino acid levels, respectively whereas these values were 87–88% and 93–96% with subgroup A isolates.

Differential ability of LBVaV and MLBVV transmission from S. vulgaris *and* S. oleraceus *to lettuce by the* O. brassicae *fungus*

The MLBVV and LBVaV transmission from *S. oleraceus*, the only naturally infected plant species found in our surveys, and *S. vulgaris* to lettuce by *O. brassicae* was investigated. Lettuce plants were selected as the positive control. *Senecio vulgaris* was included in the study not only because it is perhaps the most important weed in the family Compositae present in Spanish lettuce crops but also because of its apparent lack of susceptibility to MLBVV and LBVaV infection. A schematic representation of the experiment is shown in Figure 3. Three weeks after emergence, *S. vulgaris*, *S. oleraceus* and lettuce plants were inoculated with virulent *O. brassicae* zoospores from diseased lettuce stock plants as previously described (see Materials and methods) (Figure 3a). Two weeks later, the RS and Z of the

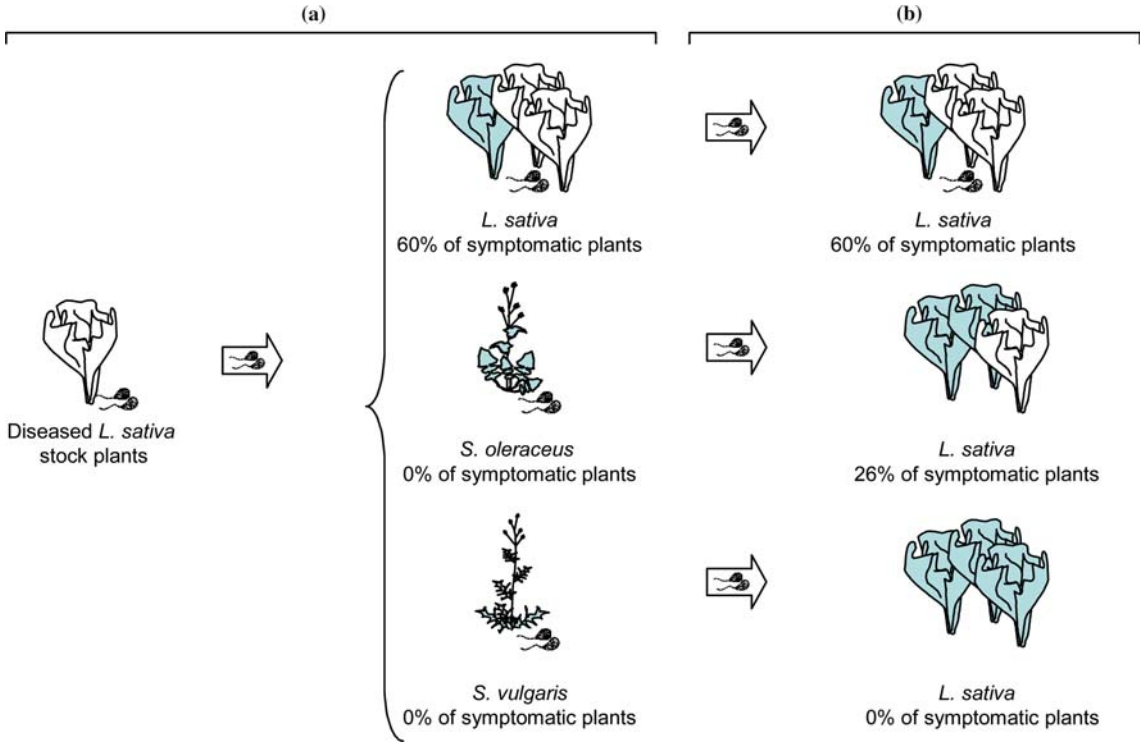

Figure 3. Schematic representation of the experiment performed to study the ability of LBVaV and MLBVV transmission from *S. oleraceus* and *S. vulgaris* to lettuce by *O. brassicae* fungus. (a) A stock of MLBVV and LBVaV diseased lettuce were used as a source of *O. brasicae* zoospores to inoculate lettuce, *S. oleraceus* and *S. vulgaris* plants. (b) After confirmation of infection of *S. oleraceus* and *S. vulgaris* roots by *O. brassicae*, zoospores were isolated and used to inoculate lettuces. Symptomatic and asymptomatic plants are represented by white and grey pictures, respectively. Percentages of symptomatic plants after 2 weeks post-inoculation are indicated in each case. Inoculation of fungus is indicated by an arrow with a picture of two zoospores inside.

fungus were detected in *S. oleraceus* roots with a mean value of 65.3 (RS + Z) per centimeter. The proportion of each reproductive structure was similar to that found in lettuce plants which were also intensively infected by *O. brassicae*. However, the number of (RS + Z) per centimeter in *S. vulgaris* roots was only 3.8 with zoosporangia being the major reproductive structure observed (RS/Z = 10). Neither zoospores nor RS were detected in the corresponding control plants of the three species which were not inoculated with the fungus. Three weeks after inoculation, 60% of the lettuce plants developed clear symptoms of big-vein confirming the virulent state of the fungus. This result was a regular value in each transmission performed from lettuce to lettuce in order to maintain the stock of diseased lettuce plants. However, neither *S. vulgaris* nor *S. oleraceus* plants showed any type of symptoms (Figure 3a).

S. vulgaris and *S. oleraceus* plants were used as source of virulent fungal zoospores to inoculate lettuce plants. Zoospore suspensions were obtained from roots of the two species of weeds and the concentration adjusted to 5×10^4 zoospores ml^{-1} to inoculate 24 lettuce seedlings. Lettuce plants from both inoculation sources showed *O. brassicae* infection when roots were analysed. Twenty-three days later, six lettuce plants (23%) inoculated with zoospores from *S. oleraceus* showed big-vein symptoms, whereas no lettuce plants inoculated with zoospores from *S. vulgaris* showed symptoms (Figure 3b).

Dot-blot molecular hybridisation analysis was performed to confirm the presence or absence of both LBVaV and MLBVV infection in the *S. oleraceus* and *S. vulgaris* plants used as a source of virulent zoospores to inoculate lettuce (Figure 4a). For this particular purpose RNAs from a mixture of leaf slices of different weed

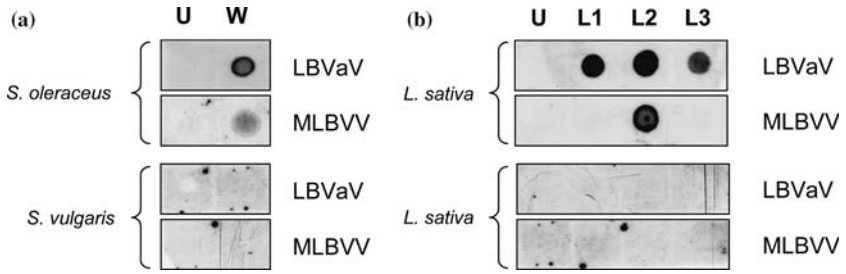

Figure 4. (a) Dot-blot molecular hybridisation to determine the presence of MLBVV and LBVaV in weed plants previously inoculated with *O. brassicae* zoospores isolated from roots of MLBVV and LBVaV infected lettuce plants. A RNA preparation from a mixture of different weed plants was used in the analysis (W). (b) *O. brassicae* zoospores isolated from *S. oleraceus* (upper) and from *S. vulgaris* (lower) were used to inoculate lettuce plants. Some of these plants (L1–L3) were subsequently analysed by dot-blot molecular hybridisation for LBVaV and MLBVV detection. U, uninfected control.

plants were extracted. Our data indicated that while in *S. oleraceus* plants both viruses could be detected in *S. vulgaris* plants, the presence of neither of the two viruses could be confirmed (lane W, Figure 4a). Furthermore, lettuce plants inoculated either with *O. brassicae* zoospores from *S. oleraceus* or from *S. vulgaris* were also analysed since the existence of symptompless plants harbouring both viruses has previously been described. Therefore, the transmission of viruses from *S. vulgaris* to lettuce could be carried out in a latent manner (Campbell, 1965). Single LBVaV in addition to mixed infection (LBVaV and MLBVV) were observed among the analysed lettuce plants which were inoculated with *O. brassicae* zoospores isolated from *S. oleraceus* plants (Figure 4b upper, lanes L1–L3). However, neither LBVaV nor MLBVV were detected in lettuce when the source of inoculum was *S. vulgaris* (Figure 4b lower, Lanes L1–L3).

Discussion

In order to reduce the spread of plant viruses it is important to know which species of the weed flora growing around economically important crops act as virus reservoirs. Identifying the species that can and cannot be infected by both LBVaV and MLBVV could be useful in the establishment of management guidelines to avoid the spread of the big vein disease. Lettuce and endive are the only cultivated plants susceptible to the infection of not only the fungus *O. brassicae* but also the two viruses associated with the disease. Several alternative host plants for *O. brassicae* that are able to transmit big-vein disease to healthy lettuce plants have already been described but only *S. oleraceus* and *S. asper* were actually associated with lettuce crops (Brunt et al., 1997). These results were based on symptom appearance after back inoculation in lettuce bait plants of the virulent fungus isolated from alternative host species. However, plants containing both viruses may or may not show symptoms since symptomatology is highly dependent on environmental conditions (Walsh, 1994). Moreover, it is possible that the fungus but not viruses infect a wide range of plant species. In this situation, the persistence of the viruses in the fungal vector may result in the spread of the disease from non-susceptible plants if rapid transmission from a fungus-infected plant takes place. Therefore, plant species could be erroneously identified as susceptible or non-susceptible by using this type of bait plant test.

In this work, the potential role as host plants for both viruses associated with big vein disease of some frequent and abundant occurring weed species in Spanish lettuce crops was studied by using dot-blot molecular hybridisation as the detection method (Navarro et al., 2004). The results showed *S. oleraceus* as the only species susceptible to LBVaV and MLBVV infection among those analysed (Table 1). Interestingly, LBVaV was individually detected in six plants while MLBVV infection was not observed alone in any plant. Co-infection of plants by both viruses was detected in four samples. Similar results have been previously reported in an infection progress analysis of both viruses in a Spanish lettuce crop (Navarro et al., 2004). Interestingly, unlike previous results

(Campbell, 1965), infected *S. oleraceus* plants did not show any type of symptoms. Very probably, as occurs in lettuce plants, *S. oleraceus* may or may not express symptoms depending on local factors such as temperature, luminosity and soil conditions (moisture and pH). Usually, low temperatures and reduced light intensity increase the severity of symptoms on lettuce (Walsh, 1994).

The identity of the isolates collected from *S. oleraceus* plants was established based on sequence analysis of the CP genes of both viruses. The low degree of genetic diversity among nucleic acid and its deduced amino acid sequences of LBVaV from lettuce and weeds in addition to the close phylogenetic relationship among Spanish lettuce and weed isolates which form a monophyletic group, suggests the capacity of these viruses to infect both natural hosts. On the other hand, MLBVV isolates from weeds were phylogenetically related to MLBVV isolates from subgroup B, with a low degree of genetic diversity among them. It is tempting to speculate that only MLBVV isolates belonging to the phylogenetic subgroup B are able to infect *S. oleraceus* plants; this could represent a host-specific phenomenon.

In order to investigate the potential capability of the two main weed species present in Spanish lettuce crops (*S. oleraceus* and *S. vulgaris*) as a source of the big-vein disease, both weed species were infected by a specific *O. brassicae* isolate obtained from Spanish lettuce. The fungal isolate obtained from these weed roots was then used to infect lettuce plants. Only the zoopores isolated from *S. oleraceus* roots remained virulent since several lettuce plants inoculated with them showed big-vein symptoms as well as the presence of both viruses. Our results suggested a different role for each of the two weed species analysed. *Sonchus oleraceus* is a compatible host of *O. brassicae* able to transmit the big-vein disease to healthy lettuce plants via the fungal vector as previously reported (Campbell, 1963, 1965; Brunt et al., 1997). In addition, we showed that this ability relied on the presence of the two viruses inside the *S. oleraceus* plants rather than to their persistence in the fungal vector. The infectivity cycle established in this research demonstrated that *S. oleraceus* can host LBVaV and MLBVV and sequence data strongly suggested that this weed can be a reservoir for both viruses. However, the infection of this weed by these two viruses could be less efficient than in lettuce plants as suggested by the small number of infected samples found in the field (Table 1) and by the low percentage of symptomatic lettuce plants obtained from the inoculation with zoospores isolated from *S. oleraceus* roots (23% vs 60%, Figure 3b). On the other hand, *S. vulgaris* did not seem to act as a reservoir but it could be considered as an intermediate host of *O. brassicae*. As a consequence, it may contribute to dilute virulent inoculum in the soil.

Rigorous weed control is an important step to take in order to reduce the incidence of viruses infecting lettuce crops. However, this strategy has to be carried out carefully since weeds also harbour beneficial predators and parasites that keep the virus and/or vector populations at low levels. We have therefore reported a method in this paper that distinguishes true and natural weed reservoirs from intermediate plants; this could be useful for diluting virulent vector concentrations and for the design of a management plan for vegetation control.

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