Transmission by *Olpidium brassicae* of *Mirafiori lettuce virus* and *Lettuce big-vein virus*, and Their Roles in Lettuce Big-Vein Etiology

Hervé Lot, Robert N. Campbell, Sylvie Souche, Robert G. Milne, and Piero Roggero

First and third authors: INRA, Pathologie Végétale, BP 94, 84143 Montfavet Cedex, France; second author: Department of Plant Pathology, University of California, Davis 95616; and fourth and fifth authors: Istituto di Fitovirologia Applicata, CNR, Strada delle Cacce 73, I-10135 Torino, Italy.

Accepted for publication 20 November 2001.

ABSTRACT

Lot, H., Campbell, R. N., Souche, S., Milne, R. G., and Roggero, P. 2002. Transmission by *Olpidium brassicae* of *Mirafiori lettuce virus* and *Lettuce big-vein virus*, and their roles in lettuce big-vein etiology. *Phytopathology* 92:288-293.

Big-vein disease occurs on lettuce worldwide in temperate conditions; the causal agent has been presumed to be *Lettuce big-vein virus* (LBVV), genus *Varicosavirus*, vectored by the soilborne fungus *Olpidium brassicae*. Recently, the role of LBVV in the etiology of big-vein disease has been questioned because a second soilborne virus, *Mirafiori lettuce virus* (MiLV), genus *Ophiovirus*, has been found frequently in big-vein-affected lettuce. LBVV and MiLV, detectable and distinguishable by enzyme-linked immunosorbent assay using specific antisera, were tested for their ability to be transmitted from lettuce to lettuce by mechanical

inoculation of sap extracts, or by zoospores of *O. brassicae*, and to cause big-vein disease. Both viruses were mechanically transmissible from lettuce to herbaceous hosts and to lettuce, but very erratically. LBVV was transmitted by *O. brassicae* but lettuce infected with only this virus never showed symptoms. MiLV was transmitted in the same manner, and lettuce infected with this virus alone consistently developed big-vein symptoms regardless of the presence or absence of LBVV. With repeated mechanical transmission, isolates of both viruses appeared to lose the ability to be vectored, and MiLV appeared to lose the ability to cause big-vein symptoms. The recovery of MiLV (Mendocino isolate, from California) from stored *O. brassicae* resting spores puts the earliest directly demonstrable existence of MiLV at 1990.

Additional keywords: fungal vector, soilborne viruses.

Understanding of the etiology of lettuce big-vein disease (BV) has developed slowly since the disease was described as possibly caused by a virus in 1934 (14). The causal agent was postulated to be a root-infecting virus transmissible without the aid of a vector (1). When *Olpidium brassicae* (Woronin) P. A. Dang. was suggested as the cause of the disease in the absence of evidence for a virus (11,12), the soilborne virus hypothesis was questioned, but it was revived with new evidence that the causal agent was graft transmitted but not mechanically transmitted, and could be separated from the obligately parasitic, soilborne fungus, *O. brassicae*, which functioned as the vector (2,6–8,19). The virus–vector relationship was recognized as the *in vivo* type in which virus-free vectors acquire the virus during the vegetative part of their life cycle in the roots of virus-infected host plants. Zoospores released from sporangia apparently carry the virus in their protoplasts and transmit it to healthy roots. The virus also enters vector resting spores, thus surviving for years in the absence of a growing host plant (2,3,5).

The first identification of a specific particle as that of *Lettuce big-vein virus* (LBVV) was provided in Japan in 1983 (15,16). The virions were rod shaped, 18 by 320 to 360 nm, with a coat protein of ≈ 48 kDa. The particles resembled those of *Tobacco stunt virus* (TStV) and reacted with antiserum to TStV. The

association of LBVV particles with BV was confirmed in Germany, where partial purification of LBVV was achieved, an antiserum was produced, and the serological relationship of LBVV with TStV was confirmed (22). The authors noted, however, that partial purifications of LBVV from lettuce with BV symptoms frequently failed to yield virions for unknown reasons.

In Holland, LBVV was mechanically transmitted to *Chenopodium quinoa* and *C. amaranticolor*, which generally developed local lesions, and to *Nicotiana benthamiana*, *N. clevelandii*, and *N. occidentalis*, which were systemically infected (13). Although LBVV virions were found in three isolates of the virus, none were found in the isolate they used most commonly. The authors demonstrated *in vivo* acquisition of a virus from mechanically inoculated *N. occidentalis*, and transfer back to lettuce that developed BV. They concluded “the mechanically transmissible local lesion agent (i.e., LBVV) was the incitant of big-vein.”

With the foregoing evidence on morphology, serology, vector relationship, and other characteristics, LBVV was designated the type virus in a newly established, unassigned genus, *Varicosavirus* (20). However, a reevaluation of the etiology of BV has become necessary since a second virus, *Mirafiori lettuce virus* (MiLV), provisionally placed in the unassigned genus *Ophiovirus*, was found to commonly coinfect field-grown lettuce showing BV (17). A few lettuce plants produced BV after MiLV was mechanically transmitted to them from sap of *C. quinoa* and *N. benthamiana*, and from partially purified preparations of *C. quinoa*. Presence of an ophiovirus in addition to the varicosavirus in big-vein diseased lettuce has been confirmed (21).

The objectives of this article are to describe transmission by mechanical inoculation and by *O. brassicae* of MiLV and LBVV, and to compare their roles in the etiology of lettuce big-vein disease. The availability of reliable and specific enzyme-linked

Corresponding author: H. Lot; E-mail address: Herve.Lot@avignon.inra.fr

*The e-Xtra logo stands for “electronic extra” and indicates the HTML abstract available on-line contains supplemental material not included in the print edition. On-line color photographs show lettuce big-vein in plants infected by *Mirafiori lettuce virus* compared with a symptomless plant infected by *Lettuce big-vein virus*.

immunosorbent assays (ELISAs) for MiLV and LBBV has been an important factor in realizing these objectives.

MATERIALS AND METHODS

Culture of *O. brassicae*. Isolation of *O. brassicae* from field-grown plants or field-collected soil has been described (4). Routine maintenance of stock cultures as well as vector transmission experiments (4) were done with lettuce plants (cv. Danilla) grown in nonsterile conditions using presterilized sand culture in growth chambers kept at 17 to 20°C with a 16-h photoperiod using mercury iodide 400W bulbs. *O. brassicae* zoospore inoculum was prepared by washing roots free from sand and immersing the roots in tap water for 10 to 15 min, at which time a drop of water was examined by phase-contrast microscopy for the presence of zoospores. The same method was used to check the presence or absence of *O. brassicae* in experimental plants each time a transfer of the fungus was made, as well as when inoculated seedlings were assayed or transplanted. The fungus was found in the hosts to which it was inoculated and absent from the controls which were not inoculated. For simplicity in presenting the results, details on the assays for *O. brassicae* will not be given in the results.

Origins of vector and viruses. The following conventions were used to identify vectors and viruses. A complex of the vector and one or more viruses obtained from field-grown plants or soil was termed a culture. When the vector was free from viruses or the viruses had been separated from the vector by mechanical transmission, each one was termed an isolate. When a virus was acquired by the vector in an acquisition trial, the culture was given a new designation with the prefix "A".

A single sporangial isolate (SS58) of *O. brassicae* was recovered in 2000 from dry roots stored in 1993 and was used for all acquisition trials. Although this isolate was made in 1987 from a big-vein-prone field in the Salinas Valley, CA, it did not transmit BV when it was made or when it was maintained on lettuce for other studies (9,10). It is a plurivorous strain compatible with lettuce but incompatible with *N. benthamiana*, in which the infecting thalli abort soon after penetration, as described for isolates of *O. bornovanus* (9).

Lettuce plants of cv. Winnie with BV symptoms were collected in Provence, France, during winter 1999–2000 and both LBBV and MiLV were mechanically transmitted from the leaves of one plant to *N. benthamiana* and *C. quinoa*. This virus mixture was

mechanically propagated on *N. benthamiana* and lettuce but only MiLV was detected after the third transfer; this isolate was labeled MiLV-Wi-1. It was transferred in nine further mechanical inoculations to plants that always tested negative for LBBV, before being used in acquisition trials. BV developed in four of four lettuce plants inoculated along with *N. benthamiana* in the fourth transfer, and in four of five lettuce plants inoculated in the fifth transfer.

A sample of roots and attached soil from the original Winnie plants was air dried and stored for 6 months, at which time a culture of *O. brassicae* was recovered and inoculated to lettuce seedlings. After 6 weeks, one plant had become ELISA-positive for both LBBV and MiLV (culture P1) and another plant tested positive only for LBBV (culture P7).

MiLV-I-47 was described by Roggero et al. (17). It was recovered from freeze-dried leaves of *C. quinoa* for the present trials by mechanical inoculation to *N. benthamiana* and maintained through six mechanical passages on this host before use in acquisition trials.

Viruliferous *O. brassicae* culture M originated from a field-grown romaine lettuce with typical BV in Mendocino County, CA. Roots and attached soil were collected and dried in 1990. When recovered for the present study in 2000, the culture produced BV on bait seedlings, which were ELISA-positive for MiLV, but not for LBBV. By electron microscopy (EM), MiLV, but not LBBV, particles were seen by negative staining of extracts of these plants, either directly or after attempted trapping with the respective antisera. This isolate was labeled MiLV-M; it was maintained in lettuce by fungal transfer until mechanically transmitted to acquisition source plants.

LBBV-Br8 originated from a butterhead lettuce with faint necrotic spots collected in Brazil by H. Lot in winter 1998. After mechanical inoculation to lettuce and *N. benthamiana*, infected plants became ELISA-positive using an LBBV antiserum provided by H. J. Vetten (22). They did not react with MiLV antiserum when it became available. Particles of LBBV, but not of MiLV, were detected by EM. LBBV-Br8 was maintained in lettuce and *N. benthamiana* by monthly mechanical transmission for nearly 2 years before use in acquisition trials.

LBBV-I-181 was isolated in May 2000 from a romaine lettuce collected in the field in Italy where MiLV-I-47 was found earlier. No MiLV was detected in this plant by EM or ELISA. The isolate was mechanically transmitted twice to *N. benthamiana* before use in acquisition trials.

Culture A-6.7 was SS58 with LBBV-I-181 acquired from a mechanically inoculated source plant (Table 1, C series).

TABLE 1. Transmission in lettuce by *Olpidium brassicae* of isolates of *Mirafiori lettuce virus* (MiLV) and *Lettuce big-vein virus* (LBBV) after repeated mechanical transfers (protocol 1)

Acquisition source plant ^u		Assay seedlings ^v		Assay transplants		
Virus isolate ^w	Replicates	MiLV	LBBV	MiLV ^x	LBBV ^x	Symptoms ^y
A series						
MiLV-Wi-1	6	–	–	NT	NT	0/32
LBBV-Br8	4	–	–	NT	NT	0/16
Controls	17	–	–	NT	NT	0/136
B series						
MiLV-I-47	3	+	–	39/48	0/48	0/48
LBBV-Br8	1	–	–	0/16	0/16	0/16
Controls	6	–	–	0/12	0/12	0/48
C series						
MiLV-I-47	5	–	–	0/24	0/24	0/40
LBBV-I-181	3	–	+ ^z	0/24	23/24	0/24
Controls	4	–	–	0/8	0/8	0/24

^u Viruses detected in leaves by enzyme-linked immunosorbent assay (ELISA) at the start of the acquisition period, and in roots 2 weeks later at the end of the acquisition period (results identical and combined).

^v Viruses detected in roots by ELISA at the end of the transmission period.

^w A, B, and C series represent different groups of trials. Controls include virus-infected plants without vector, vector-infected plants without virus, and non-inoculated plants.

^x Number of transplants positive by ELISA/number of transplants tested from all replicates. NT = not tested, due to negative results with assay seedlings.

^y Number of transplants with big-vein disease symptoms/number of transplants in all replicates.

^z Three viruliferous cultures were labeled and kept as A6.7 to A6.9.

Mechanical transmission. Leaf tissue was homogenized (1:3, wt/vol) with 0.03 M disodium phosphate, pH 8.3, containing 0.2% sodium diethyldithiocarbamate (DIECA) and activated charcoal at 100 mg/ml. Source leaves, mortars, and solution were cooled on

ice. Test plants were raised in a cooled greenhouse but when temperatures could not be held below 18°C, lettuce plants were kept in a growth chamber as specified previously. Generally, five plants of each species were inoculated, but 10 lettuce seedlings were used in some assays. Plants were observed for symptoms and tested for virus infection with ELISA.

Electron microscopy. Extracts of some plants were checked by EM after negative staining with 1% (wt/vol) aqueous uranyl acetate. Immunosorbent electron microscopy (ISEM) employing standard procedures was also used.

Virus acquisition and transmission by the vector. Although acquisition and transmission of viruses by fungi are distinct processes, we could not test acquisition other than by subsequent transmission, so for convenience, "transmission" as used below includes acquisition.

Transmissibility of LBBV and MiLV to lettuce by *O. brassicae* was tested using three protocols and virus isolates that had been mechanically transmitted several times or only once or were in viruliferous cultures of the fungus.

In protocol 1, lettuce seedlings were transplanted into sand in 200-ml pots and grown for ≈3 weeks. These candidate acquisition source plants were mechanically inoculated on two to three young, fully formed leaves using care to avoid dripping inoculum onto the sand, and grown for ≈3 more weeks. At the five- to seven-leaf stage, a composite leaf sample from each plant was tested for each virus by ELISA. Only plants giving a positive reaction were kept as acquisition source plants. They were placed in individual saucers to prevent cross-contamination, and inoculated with zoospores of *O. brassicae*-SS58 or used as vector-free controls. The acquisition period generally lasted 2 weeks, at which time the vector was recovered in a root washing that was checked for zoospores, and 20 ml was inoculated to 15 to 20 assay seedlings sown 8 to 10 days earlier in a 100-ml pot. At this time, the roots of the acquisition source plants were tested by ELISA for both viruses. The pots were incubated for a 2-week transmission period, at which time eight seedlings (termed "assay transplants") were carefully removed and repotted individually. The roots of the remaining assay seedlings were tested, as a group, by ELISA. The assay transplants were grown in 200-ml pots of sand if the plants were to be incubated in growth chambers, with strict precautions against splash contamination. Alternatively, transplants incubated in a greenhouse in the fall and winter were raised in 11-by-11-cm pots, with minimum night temperatures of 4 to 6°C. The pots were placed on metal frames on soilless benches to prevent capillary uptake of water from the bench. Greenhouse plants were irrigated carefully to minimize cross contamination. The minimum incubation period for BV expression by assay transplants was 3 to 4 weeks, and all plants were observed for 7 to 10 weeks. Pieces of two or three leaves of each plant were removed and tested by ELISA for both viruses one to three times before the trial ended.

In protocol 2, the acquisition source plants were seedlings germinated for 1 to 2 weeks in a pot of sand, then inoculated with SS58 zoospores, transplanted individually to pots of sand a few days later, and grown for ≈3 weeks, at which time they were individually mechanically inoculated with virus or viruses. After an acquisition period of ≈3 weeks, the leaves were tested by ELISA, and those plants that were virus infected were used as acquisition source plants. A few days later, zoospores were harvested from each source plant and inoculated to assay seedlings in two pots. After a transmission period of ≈2 weeks, the assay transplants were repotted and grown further, while the roots of the remaining seedlings were pooled and tested by ELISA as in protocol 1.

In protocol 3, seedlings in two pots were directly inoculated with each culture of *O. brassicae*. After a 12-day transmission period, a total of 24 seedlings from each treatment were transplanted and transferred to the greenhouse. Observation of symptoms and virus detection by ELISA were done as before.

Fig. 1. A, Particles of *Lettuce big-vein virus* (isolate BR-8) from crude sap of lettuce infected by mechanical inoculation; note rigid rods with wide axial canal and evident helical repeat. B, Particles of *Mirafiori lettuce virus* partially purified from naturally infected field lettuce in Italy; note a smaller particle (above) and a larger particle, both formed of fine supercoiled threads; the very small circular particles are host plant Rubisco (D-ribulose 1,5-biphosphate carboxylase-oxygenase, RuBP). Both viruses imaged in 1% aqueous uranyl acetate at the same enlargement. Bar = 100 nm.

In early trials, three types of control were maintained: (a) virus-infected without fungal vector, (b) vector-infected without virus, and (c) noninoculated, with one pot of each type of control for each acquisition source plant. This design required too much space and, in later trials, only one pot of each type of control was kept in a given trial, or only the nonviruliferous vector control was used. These controls were tested along with the plants in the main treatment and always remained in the condition specified: controls a and c were not contaminated with the vector and controls b and c were not infected with viruses. These controls will not be mentioned in the text.

Detection of MiLV and LBVV by ELISA. The presence of MiLV and LBVV always was tested by ELISA using specific rabbit antisera. ELISA for MiLV has been described (17). The antiserum did not cross-react with LBVV, as shown by ELISA, western blotting, EM decoration, and ISEM tests. An antiserum to LBVV-Br8 was prepared with virus purified after 6 to 10 mechanical passages in *N. benthamiana* (H. Lot, unpublished data). We detected no cross-reaction with MiLV by ELISA, western blotting, or EM; we found the same specificity as in the antiserum to LBVV prepared by H. J. Vetten (22).

The antisera were cross-absorbed with preparations from healthy plants, and the immunoglobulin G fractions, purified by affinity chromatography, were conjugated with alkaline phosphatase. Double-antibody sandwich-ELISA was performed by standard procedures. Lettuce leaf samples were diluted 1:5 (wt/vol) with PBS-Tween containing 2% PVP and 0.2% DIECA, whereas root samples were diluted 1:10 in the same buffer. Samples were considered positive if absorbance was more than three times that of the corresponding healthy controls of the same tissue.

RESULTS

Mechanical transmission. Both MiLV and LBVV were mechanically transmitted to lettuce, *N. benthamiana*, *N. clevelandii*, *N. occidentalis*, and *C. quinoa*, but erratically and with low rates of infection (data not shown).

MiLV isolates Wi-1, I-47, and M systemically infected *N. benthamiana*, *N. clevelandii*, and *N. occidentalis*, as determined by ELISA; on *C. quinoa*, all isolates gave only local lesions. On lettuce, Wi-1 produced BV up to the fifth mechanical passage but thereafter the plants were symptomless, even though infected as determined by ELISA. Isolate I-47 initially produced BV (17) but not when back inoculated to lettuce after six mechanical passages in *N. benthamiana*. MiLV-M produced BV every time it was mechanically transferred to lettuce from the vector-maintained culture.

LBVV isolates Br8 and I-181 gave results similar to MiLV; namely, systemic infections on the three *Nicotiana* spp. and local infection on *C. quinoa*. In lettuce, infection was systemic, as determined by ELISA, but symptomless.

Viral morphology. The viruses are detectable and distinguishable by EM. LBVV particles are fragile, rather rigid rods 320 to 360 nm in length and 18 nm in diameter, with a central canal and an obvious helix of pitch ≈ 5 nm (Fig. 1A) (20,22). MiLV particles, like those of recognized ophiioviruses, are highly kinked filaments ≈ 3 nm in diameter that form masses of two distinct sizes but of undetermined contour length; they probably form closed circles, because free ends are very seldom seen (Fig. 1B) (12,20).

In crude sap of infected lettuce, small numbers of LBVV virions are detectable with difficulty, but MiLV particles are not detectable. Both viruses are detectable more readily in the crude sap of some systemically infected test plants, such as *N. benthamiana*. With ISEM, both viruses are readily found in all susceptible plants, including lettuce. Even when present, MiLV particles are not easily noted because they have low contrast and vary in degree of coiling and in size; thus, in contrast to LBVV, they do not look like "a typical virus-like particle," but may be taken for host debris.

TABLE 3. Transmission in lettuce by *Olipidium brassicae* of *Mirafiori lettuce virus* (MiLV-M) after one mechanical transfer (protocol 2)¹

Acquisition source plant		Assay seedlings		Assay transplants ^u	
Number	ELISA ^v	Pot no.	ELISA ^w	ELISA ^x	Symptoms ^y
1	+	1-A	—	0/8	0/8
		1-B	—	0/8	0/8
2	+	2-A	+	8/8	8/8
		2-B	+	1/8	1/8
3	+	3-A	—	0/8	0/8
		3-B	—	0/8	0/8
4	+	4-A	+	8/8	8/8
		4-B	+	6/8	6/8
5	+	5-A	+	8/8	8/8
		5-B	+	2/8	2/8
6	+	6-A	+	8/8	8/8
		6-B	+	4/8	4/8
Control ^z	—	Control-A	—	0/8	0/8
		Control-B	—	0/8	0/8

¹ Acquisition source plants, assay seedlings, and assay transplants always tested negative for *Lettuce big-vein virus* by enzyme-linked immunosorbent assay (ELISA) (data not shown).

^u Transplantation was done at the end of the transmission period. Seedlings from pot A were maintained in a growth chamber at 17 to 20°C. Seedlings from pot B were transferred to a greenhouse with daily maximum temperatures above 18°C.

^v MiLV detection in leaves 26 days after mechanical inoculation and in roots 3 days later at the end of the acquisition period (results identical and combined).

^w MiLV detection in roots by ELISA at the end of the transmission period.

^x Number of transplants positive for MiLV/number of transplants.

^y Number of transplants with big-vein disease symptoms/number of transplants.

^z Plant inoculated with SS58 (nonviruliferous).

TABLE 2. Transmission in lettuce by *Olipidium brassicae* of *Mirafiori lettuce virus* (MiLV) and *Lettuce big-vein virus* (LBVV) isolates after one mechanical transfer (protocol 1)

Acquisition source plant ^v			Assay seedlings ^w		Assay transplants		
Plant number	MiLV	LBVV	MiLV	LBVV	MiLV ^x	LBVV ^x	Symptoms ^y
1	+	—	+	—	16/16	0/16	16/16
2	+	—	+	—	16/16	0/16	16/16
3	+	—	+	—	16/16	0/16	16/16
4	—	+	—	+	0/16	16/16	0/16
Mixture ^z	...	...	+	+	8/8	6/8	8/8
Control	—	—	—	—	0/8	0/8	0/8

^v Viruses detected in leaves by enzyme-linked immunosorbent assay (ELISA) 19 days after mechanical inoculation, and in roots 2 weeks later at the end of the acquisition period (results identical and combined).

^w Viruses detected in roots by ELISA at the end of the transmission period.

^x Number of transplants positive by ELISA/number of transplants tested.

^y Number of transplants with big-vein disease symptoms/number of transplants tested.

^z Mixture consisted of equal volumes of zoospore suspension from plants 1 through 4 inoculated to assay seedlings at the start of the transmission period. Control = plants inoculated with SS58 (nonviruliferous).

Transmission by *O. brassicae* of MiLV and LBVV isolates after repeated mechanical transmission. Isolates MiLV-Wi-1 and LBVV-Br8, both maintained for several months by mechanical transfer, were not fungus transmitted in three trials using protocol 1 (Table 1, A series). LBVV-Br8 was not transmitted in another trial (Table 1, B series). Another mechanically passaged isolate, MiLV I-47, was transmitted by *O. brassicae* from each of three acquisition source plants (Table 1, B series) but none of the assay transplants developed BV, although MiLV was detected in 39 of 48 transplants. However, MiLV I-47, which was mechanically inoculated one more time, was not transmitted by *O. brassicae* from five acquisition source plants (Table 1, C series). In the same series, LBVV-I-181 was transmitted to 23 of 24 plants but no symptoms developed.

We concluded that the viruses maintained by repeated mechanical transfer may lose the ability to cause symptoms and to be vector transmitted.

Transmission by *O. brassicae* of MiLV and LBVV isolates after a single mechanical transmission. In attempts to obtain more consistent results, we used viral inocula originating directly from the field or from vector-maintained cultures. We mechanically inoculated directly from each of three field-collected plants with BV to 15 candidate acquisition source plants in which *O. brassicae* was already established (protocol 2). These three plants were taken from the field where MiLV-I-47 was collected and were selected because they had a relatively high titer of one virus and a low titer of the other. It was hoped that some plants would be infected by a single virus in this mechanical transfer. Indeed, 4 of 45 plants became systemically infected by LBVV but none by MiLV. The LBVV in each of the four acquisition source plants was transmitted by *O. brassicae*. None of 32 assay transplants developed BV symptoms, although LBVV was detected in the leaves of 28 plants.

In another trial, MiLV-M and LBVV-A6.7 from vector-inoculated lettuce plants were mechanically inoculated to candidate acquisition source plants (protocol 1). The plant from which MiLV-M was transmitted had BV symptoms but the plant from which LBVV-A6.7 was transmitted did not. Three of nine plants became infected by MiLV-M and these developed BV by the end of the acquisition period; 1 of 12 plants became infected with LBVV-A6.7 but this plant did not express symptoms. When zoospores were transferred from these acquisition hosts to assay seedlings, an extra treatment was added by inoculating a single pot of assay seedlings with a zoospore mixture containing 5 ml of the root washing from each of the four source plants (Table 2). All the assay transplants inoculated with MiLV-M alone became ELISA-positive for MiLV, but not for LBVV, and all developed BV. All plants inoculated with LBVV-A6.7 alone became positive only for LBVV, and all remained symptomless. Most plants inoculated with the mixture became positive for both viruses and developed BV symptoms that did not differ from those caused by MiLV alone.

In a further trial using protocol 2, MiLV-M was mechanically inoculated to 20 candidate acquisition plants, of which 6 became

infected and expressed BV by the end of the acquisition period, which was increased to 4 weeks. Zoospores of *O. brassicae* from each source were inoculated to assay seedlings in two series of pots (A and B). The virus was transmitted from four of six source plants (Table 3). The assay transplants in the A series were incubated in a growth chamber; all 32 plants developed BV, mostly within 5 weeks, and became ELISA-positive for MiLV. The B transplants were incubated in a greenhouse where peak temperatures rose above 18°C. Only 14 of these 32 plants expressed BV and only these 14 plants were positive for MiLV. Symptoms appeared over a later, longer period at 5 to 7 weeks.

Direct inoculation using *O. brassicae* carrying MiLV or LBVV. The roles of MiLV and LBVV in BV disease also were tested by inoculating viruliferous cultures of *O. brassicae* directly to assay seedlings (protocol 3). These cultures contained MiLV-M alone, LBVV (A6.7 or P7) alone, both viruses in one culture (P1), or a mixture of zoospores from two cultures (MiLV-M plus LBVV-A6.7). Each culture was inoculated to assay seedlings in two pots. After a 12-day transmission period, 24 assay transplants were repotted from the two pots of assay seedlings and transferred to the greenhouse (maximum temperatures not exceeding 18°C). The viruliferous cultures transmitted their respective viruses to the assay seedlings as expected (Table 4). Every transplant inoculated with MiLV-M alone developed BV and was ELISA-positive for MiLV. Of 24 plants inoculated with the MiLV-M plus LBVV-A6.7 mixture, 15 were positive for both viruses and developed BV symptoms that were not different from those with MiLV-M alone. Symptoms may have developed less uniformly in this treatment because the MiLV-M inoculum was diluted. All 24 plants inoculated with culture P1 tested positive for both viruses and showed BV symptoms that developed as uniformly as with MiLV-M alone. On the other hand, none of 57 plants infected by LBVV alone developed BV.

DISCUSSION

Our results establish for the first time the roles played by LBVV and MiLV in the etiology of lettuce BV. Both viruses are transmitted by *O. brassicae*, which acquires each virus in the in vivo manner. This is the first vector identified for an *Ophiovirus*, and the first demonstration that LBVV is vectored by *O. brassicae*.

Roggero et al. (17) showed that partially purified preparations of MiLV I-47 could induce BV after mechanical transmission. Our results confirm and extend that result by showing that MiLV regularly causes BV following vector transmission to seedlings. On the other hand, LBVV appears to play no etiological role, infecting lettuce without causing symptoms and not influencing the outcome of MiLV infection. The possibility that the LBVV isolates were attenuated through mechanical passaging and thus unable to induce BV is unlikely, because LBVV isolates that were not extensively passaged did not induce BV (i.e., those from fungal isolates P7 and I-181 and the numberless LBVV isolates).

TABLE 4. Ability of *Mirafiori lettuce virus* (MiLV) and *Lettuce big-vein virus* (LBVV) isolates to cause big-vein disease (BV) symptoms on lettuce after direct inoculation with viruliferous *Olipidium brassicae* cultures

<i>O. brassicae</i> culture	Virus present ^x		Assay transplants			
	MiLV	LBVV	MiLV alone ^y	LBVV alone ^y	MiLV + LBVV ^y	Symptoms ^z
M	+	—	24/24	0/24	0/24	24/24
A 6.7	—	+	0/24	24/24	0/24	0/24
M + A 6.7 mixed	+	+	0/24	9/24	15/24	15/24
P1	+	+	0/24	0/24	24/24	24/24
P7	—	+	0/24	24/24	0/24	0/24
SS58 (nonviruliferous control)	—	—	0/24	0/24	0/24	0/24

^x Viruses detected by enzyme-linked immunosorbent assay (ELISA) in vector source plants and in assay seedlings (results identical and combined).

^y Number of transplants positive by ELISA for one or two viruses 7 weeks after inoculation/number of transplants.

^z Number of transplants with BV symptoms/number of transplants.

Therefore, the name “lettuce big-vein virus” has been applied erroneously to a virus that does not cause big-vein of lettuce. Furthermore, the genus name *Varicosavirus*, referring to vein enlargement, is now seen to be inappropriate.

Both viruses are mechanically transmissible from lettuce to herbaceous hosts and to lettuce, although with variable success. The reactions of *N. benthamiana*, *N. clevelandii*, *N. occidentalis*, and *C. quinoa* are similar for the two viruses. With repeated mechanical transfers, both viruses appeared to lose the ability to be vector transmitted, and MiLV, when transmitted, appeared to lose the ability to cause BV. Similar changes are reported for other viruses with a fungal vector (5).

Evaluation of earlier studies on BV is complicated by what must have been the frequent undetected presence of two viruses (where only one was presumed) in field-collected material and in mechanically or vector-inoculated host plants. Many early studies were limited to assays based on vector inoculation followed by symptom expression; our results indicate that MiLV was present in all these plants that developed BV, but LBBV also could have been present. More recently, LBBV was observed by EM, detected serologically, or transmitted mechanically by several groups (13,15,16,22), but only one claim of fulfilling Koch’s postulates has been made (13). This claim also must have been based on dual infection; LBBV virions were observed in all but one isolate, which likely had only MiLV, a virus more difficult to visualize by EM negative staining than LBBV (17). Similarly, Sasaya et al. (18) used lettuce plants expressing BV to purify LBBV and sequence its coat protein gene, believing that LBBV had caused the symptom, and not suspecting the additional presence of MiLV.

The recovery of MiLV-M from stored vector resting spores puts the earliest, directly demonstrable existence of MiLV at 1990. No doubt it must have been present at least since BV was first described in 1934.

ACKNOWLEDGMENTS

We thank B. Delecalle and V. Masenga for EM observations, M. Duteil and R. Lenzi for technical assistance, and H. J. Vetten for providing a sample of his LBBV antiserum.

LITERATURE CITED

1. Allen, M. W. 1948. Relation of soil fumigation, nematodes, and inoculation technique to big vein disease of lettuce. *Phytopathology* 38:612-628.
2. Campbell, R. N. 1962. Relationship between the lettuce big-vein virus and its vector, *Olpidium brassicae*. *Nature (Lond.)* 195:675-677.
3. Campbell, R. N. 1985. Longevity of *Olpidium brassicae* in air-dry soil and

- the persistence of the lettuce big-vein agent. *Can. J. Bot.* 63:2288-2289.
4. Campbell, R. N. 1988. Cultural characteristics and manipulative methods. Pages 153-165 in: *Viruses with Fungal Vectors*. J. I. Cooper and M. J. C. Asher, eds. Assoc. Appl. Biol., Wellesbourne, UK.
5. Campbell, R. N. 1996. Fungal transmission of plant viruses. *Annu. Rev. Phytopathol.* 34:87-108.
6. Campbell, R. N., and Grogan, R. G. 1963. Big-vein virus of lettuce and its transmission by *Olpidium brassicae*. *Phytopathology* 53:252-259.
7. Campbell, R. N., and Grogan, R. G. 1964. Acquisition and transmission of lettuce big-vein virus by *Olpidium brassicae*. *Phytopathology* 54:681-690.
8. Campbell, R. N., Grogan, R. G., and Purcifull, D. E. 1961. Graft transmission of big vein of lettuce. *Virology* 15:82-85.
9. Campbell, R. N., and Sim, S. T. 1994. Host specificity and nomenclature of *Olpidium bornovanus* (= *Olpidium radicale*) and comparisons to *Olpidium brassicae*. *Can. J. Bot.* 72:1136-1143.
10. Campbell, R. N., Sim, S. T., and Lecoq, H. 1995. Virus transmission by host-specific strains of *Olpidium bornovanus* and *Olpidium brassicae*. *Eur. J. Plant Pathol.* 101:273-282.
11. Fry, P. R. 1958. The relationship of *Olpidium brassicae* (Wor.) Dang. to the big-vein disease of lettuce. *N. Z. J. Agric. Res.* 1:301-304.
12. Grogan, R. G., Zink, F. W., Hewitt, W. B., and Kimble, K. A. 1958. The association of *Olpidium* with the big-vein disease of lettuce. *Phytopathology* 48:292-296.
13. Huijberts, N., Blystad, D. R., and Bos, L. 1990. Lettuce big-vein virus: Mechanical transmission and relationships to tobacco stunt virus. *Ann. Appl. Biol.* 116:463-475.
14. Jagger, I. C., and Chandler, N. 1934. Big vein, a disease of lettuce. *Phytopathology* 24:1253-1256.
15. Kuwata, S., and Kubo, S. 1984. Properties of two *Olpidium*-transmitted viruses: Tobacco stunt and lettuce big vein. 6th Int. Congr. Virol. Sendai 1984:331.
16. Kuwata, S., Kubo, S., Yamashita, S., and Doi, Y. 1983. Rod-shaped particles, a probable entity of lettuce big vein virus. *Ann. Phytopathol. Soc. Jpn.* 49:246-251.
17. Roggero, P., Ciuffo, M., Vaira, A. M., Accotto, G. P., Masenga, V., and Milne, R. G. 2000. An Ophiovirus isolated from lettuce with big-vein symptoms. *Arch. Virol.* 145:2629-2642.
18. Sasaya, T., Ishikawa, K., and Koganezawa, H. 2001. Nucleotide sequence of the coat protein gene of Lettuce big-vein virus. *J. Gen. Virol.* 82:1509-1515.
19. Tomlinson, J. A., and Garrett, R. G. 1964. Studies on the lettuce big-vein virus and its vector *Olpidium brassicae* (Wor.) Dang. *Ann. Appl. Biol.* 54:45-61.
20. van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R., and Wickner, R. B. 2000. *Virus Taxonomy: Classification and Nomenclature of Viruses*. (7th Report ICTV) Academic Press, San Diego, CA.
21. Verbeek, M., van der Wilk, F., Vetten, H. J., and van den Heuvel, J. F. J. M. 2001. Viruses associated with the lettuce big-vein syndrome. *Arbeitskreis Viruskrankheiten der Pflanzen Tagung, Koln.*
22. Vetten, H. J., Lesemann, D. E., and Dalchow, J. 1987. Electron microscopical and serological detection of virus-like particles associated with lettuce big vein disease. *J. Phytopathol.* 120:53-59.