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Occurrence of Mirafiori lettuce virus and *Lettuce big-vein virus* in relation to development of big-vein symptoms in lettuce crops

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Abstract

Big-vein disease (BV) of lettuce has been attributed to infection by *Lettuce big-vein virus* (LBVV), vectored by the soil fungus *Olpidium brassicae*. The finding of a second soil-borne virus in lettuce, Mirafiori lettuce virus (MiLV), led to a re-investigation of the role of LBVV in big-vein disease, with evidence emerging that both MiLV and LBVV are vectored by *O. brassicae*, and that MiLV, not LBVV, is the cause of BV (Lot et al. (2002), Phytopathology 92: 288–293). The two viruses have coat proteins of similar size but have different morphologies and are serologically unrelated. We tested individual lettuce plants in BV-prone fields and protected crops in France and Italy for the presence of the two viruses, using DAS-ELISA and antisera specific for each virus. Both MiLV and LBVV were found at high incidence, often together but sometimes separately. Symptoms were frequently found to be associated with MiLV alone or both viruses, but rarely LBVV alone. However, no absolute correlation emerged, because sometimes MiLV was present in the absence of symptoms, and vice versa. To clarify the situation, individual lettuce plants were examined over a period of time in two further surveys. In surveys of protected crops in France, plants with big-vein were always ELISA-positive for MiLV, or else symptomless plants positive for MiLV were later seen to develop big-vein symptoms. Presence or absence of LBVV appeared to have no effect on symptom development. In surveys of open fields in Italy, all combinations were found: presence of both viruses, apparent absence of both viruses, or presence of each one alone, in plants that developed BV. At the end of the observation period, nearly all plants had BV and contained both viruses.

Introduction

Lettuce big-vein (BV), first described in California (Jagger and Chandler, 1934), is a soil-borne disease found worldwide in all lettuce types grown in the open or under cover, and in hydroponic culture. Symptoms are severe when air temperatures are below 18 °C, but are not expressed at temperatures above 22 °C (Westerlund et al., 1978; Walsh, 1994). The economic importance of BV is due to the foliage symptoms and a reduction in head size, both limiting the proportion of marketable heads. Acceptable levels of resistance are not currently available (Falk, 1997; Bos and Huijberts, 1990), and soil sterilization with methyl bromide, in any case very costly, will soon be prohibited.

BV is transmitted by *Olpidium brassicae* (Campbell and Grogan, 1964), and is generally assumed to be caused by *Lettuce big-vein virus* (LBVV, genus *Varicosavirus*), a virus with rigid rod-shaped particles (Kuwata et al., 1983; Kuwata and Kubo, 1984; Vetten et al., 1987; Huijberts et al., 1990; Mayo, 2000). However, another virus, Mirafiori lettuce virus (MiLV; not yet officially recognized but ascribable to the genus *Ophiovirus*), was recently isolated from lettuce with BV, shown to be soilborne, and shown to induce BV in a few lettuce plants following mechanical inoculation (Roggero et al., 2000). Recent evidence indicates

that both viruses are transmitted by *O. brassicae*, and that MiLV, not LBVV, causes BV (Lot et al., 2002).

We report on methods to distinguish and detect LBVV and MiLV, and the results of ELISA screening for these two viruses in relation to BV symptom expression in lettuce where the disease is common, either protected or in open fields, in France and Italy.

Materials and methods

Recognition of big-vein symptoms

BV symptoms have often been described (see Campbell and Grogan, 1964; Tomlinson and Garrett, 1964; Vetten et al., 1987; Falk, 1997; Revers et al., 1997; Lot et al., 2002). However, specific recognition of the symptom can be influenced by the cultivar, the temperature, presence of *Lettuce mosaic virus* (LMV), and other factors. The range of symptoms that were used to identify BV in this work are indicated in the Results.

Virus isolates

MiLV isolate I-47 has been described (Roggero et al., 2000). LBVV isolate Br8 came from a butterhead lettuce with faint necrotic spots collected by HL in Brazil in 1998 (Lot et al., 2002). These isolates are further discussed in Lot et al. (2002). Mechanical inoculation to lettuce and *Nicotiana benthamiana* led to systemic infection by an isolate which was ELISA-positive using an antiserum (LBVV 557) prepared by H.J. Vetten (Vetten et al., 1987), but negative with the MiLV antiserum. The MiLV and LBVV isolates were maintained in lettuce and *N. benthamiana* by mechanical inoculation.

Antisera

The MiLV antiserum used was described in Roggero et al. (2000). A rabbit antiserum against LBVV Br8 was prepared after purification of the virus from *N. benthamiana* (H. Lot and B. Delecolle, unpublished). In the present work, the anti-BR8 serum was used, but preliminary tests were made with LBVV 557. All antisera were cross-absorbed with preparations from healthy plants. IgG fractions were prepared by affinity chromatography using protein A or protein G, and aliquots were conjugated with alkaline phosphatase.

The antiserum to LMV was prepared by HL and was used on many previous occasions (e.g. Revers et al., 1997).

DAS-ELISA

This was done by standard procedures, using coating antibodies at 1 μg/ml and conjugates diluted 1/1000. A composite sample of about 1 g was taken from at least three leaves of each lettuce plant and homogenized with PBS-Tween containing 2% PVP and 0.2% Na-DIECA (1/5, w/v). Samples were considered positive if absorbance was more than three times that of the corresponding healthy controls.

Western blots

Electrophoresis and Western blotting were done according to Roggero et al. (2000). Total protein extracts of healthy and infected leaves were denatured by boiling in Laemmli sample buffer.

Crop surveys of virus infection in relation to BV symptoms

Surveys of mature lettuce crops grown under cover at six sites were made in France in January 1999. The lettuce types were butterhead, batavia, romaine and oak-leaf. At each sample site, five plants with BV and one symptomless plant were taken and analyzed by ELISA for presence of MiLV and LBVV. Similar surveys were made of mature or almost mature field lettuce crops in several localities in Piedmont, Italy. In one survey in October–November 1999, 114 plants were scored for symptoms and analyzed by ELISA. In a second survey in April 2000, 170 plants were analyzed; they came from the field where MiLV I-47 originated, near Torino. In a third survey in November 2000, 96 plants from the same field were analyzed. Lettuce is grown year-round in this field, which has for many years been prone to BV.

Time-course analyses of infection in relation to symptoms

In France, a plastic tunnel of butterhead lettuce cv. Sagesse, near Perpignan, was chosen because by

the end of November 2000, 10% of the plants had BV symptoms. In a first survey (November 29), 20 plants with symptoms and 20 plants without were labeled, and leaf samples were taken for ELISA. In a second survey on December 20, all the plants were again scored for symptoms and sampled for ELISA. They were further observed for symptoms until January 5. Day temperatures in both field and greenhouse were rarely above 20 °C over this period.

In Italy, a time-course analysis was made in a plot of romaine lettuce from the field surveyed in April 2000. Procedures were the same as in the French analysis. A first survey was done on April 4, 2001, a second on April 18, and a final symptom survey was done on May 9. During this period, day temperatures rarely exceeded 20 °C and night temperatures were around 10 °C.

Table 1. Homologous and heterologous reactions in DAS-ELISA of antisera to MiLV and LBVV

Virus isolate/host[b]	Antisera[a]		
	MiLV I-47	LBVV BR8	LBVV 557
MiLV I-47/*Nicotiana benthamiana*	2.310	0.024	0.012
MiLV I-47/lettuce	1.546	0.009	0.015
LBVV BR8/ *N. benthamiana*	0.006	1.326	0.455
LBVV BR8/lettuce	0.004	1.242	0.517
Healthy *N. benthamiana*	0.005	0.02	0.004
Healthy lettuce	0.001	0.008	0.012

[a]Plates were coated with 1 μg/ml of purified antibodies and the alkaline phosphatase conjugated antibodies were diluted 1/1000. Absorbance at 405 nm was measured after 1 h of substrate incubation.
[b]Plant sap was diluted 1/5 (w/v) with PBS-Tween containing PVP and Na-DIECA.

Results

Recognition of symptoms

Both in the open and in protected crops, the main symptoms of BV are chlorotic or white vein-banding, with crinkling and leaf distortion. Vein-banding and crinkling are very distinct on butterhead and batavia types, but crinkling is less obvious on romaine types. On oak-leaf types, especially red ones, vein-banding is not very distinct, but leaf distortion and crinkling are good guides to BV recognition. Early BV symptoms may be confused with vein clearing caused by LMV, therefore all lettuce plants analyzed in our surveys were tested for presence of this virus. Very few plants were ELISA-positive for LMV, and these were eliminated from the surveys.

Specificity of the antisera

ELISAs gave high absorbances with samples infected by the homologous viruses, with low backgrounds from healthy controls; MiLV antiserum reacted only with MiLV, and the two LBVV antisera reacted only with LBVV (Table 1). In Western blots, the antisera against MiLV and LBVV reacted only with the homologous viruses, thus confirming the ELISA results. A band of about 48 kDa was detected in each cases (Figure 1).

Crop surveys

No differences in incidences of LBVV and MiLV in relation to symptoms were found among the different types of lettuce analyzed (not shown). Results from the crop survey in different localities in France are

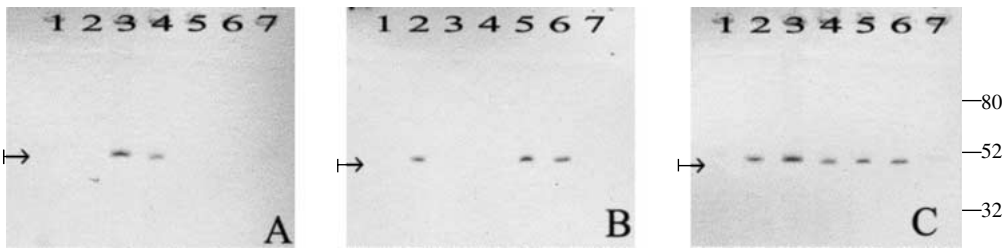

Figure 1. Western blot analysis of MiLV and LBVV after migration in SDS-PAGE of crude saps from plants infected with one or the other virus, as assayed by ELISA. Panel A, LBVV antiserum; panel B, MiLV antiserum; panel C, blot A after probing also with MiLV antiserum. Lane 1, healthy *Chenopodium quinoa*; lane 2, MiLV-infected *C. quinoa*; lanes 3 and 4, two LBVV-infected lettuces; lanes 5 and 6, two MiLV-infected lettuces; lane 7, healthy lettuce. Arrows indicate a band at about 48 kDa. On the right side, the location of MW-marker proteins is indicated with the respective molecular weight in kiloDaltons.

Table 2. ELISA detection of MiLV and LBVV in lettuces under cover showing BV or symptomless at different sites in southern France in January 1999

Lettuce type	Site	BV symptoms	MiLV alone	LBVV alone	MiLV+ LBVV	No virus
Butterhead	Carpentras	Yes	—	—	5	—
		No	—	1	—	—
Batavia	Marseille	Yes	2	—	3	—
		No	—	—	—	1
Romaine	Perpignan	Yes	—	—	5	—
		No	1	—	—	—
Oak leaf	Marseille	Yes	—	—	5	—
		No	1	—	—	—
Oak leaf	Avignon	Yes	—	—	5	—
		No	—	1	—	—
Oak leaf	Bordeaux	Yes	1	—	4	—
		No	—	—	—	1

Table 3. ELISA detection of MiLV and LBVV in lettuces in open fields showing BV or symptomless from different sites in Piedmont

BV symptoms	Number of plants	MiLV alone	LBVV alone	MiLV+ LBVV	No virus
Assay 1[a]					
Yes	69	18	11	37	3
No	45	11	2	0	32
Assay 2[b]					
Yes	81	31	4	26	20
No	89	10	19	20	40
Assay 3[c]					
Yes	50	4	6	40	0
No	46	4	14	28	0

[a]Samples collected in October–November 1999 in different localities.
[b]Samples collected in April 2000 in a single field near Torino.
[c]Samples collected in November 2000 in the same field as above.

summarized in Table 2. Of the 30 plants showing BV when analyzed by ELISA, 27 plants were found to be infected with both MiLV and LBVV, and three with MiLV alone. None were found infected by LBVV alone. Of the six symptomless plants, two were positive for MiLV, two were positive for LBVV, and two were negative for both viruses.

Results from the three crop surveys in Piedmont, Italy, are summarized in Table 3. In different plants, all possible combinations were encountered, i.e. both viruses together, one of them alone, or neither, in plants that showed symptoms or appeared healthy. In plants with BV, the proportion of plants found infected with MiLV alone or the mixture was high (80%, 70% and 88%, respectively in the three surveys) but not consistently different from that found with plants positive for LBVV alone or the mixture (70%, 37% and 90%).

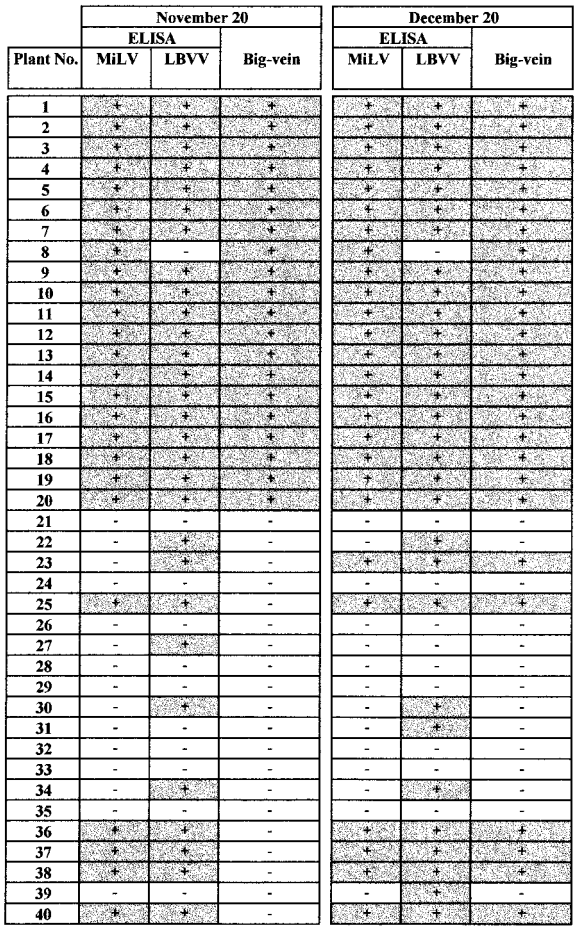

Figure 2. Detection of MiLV and LBVV, and development of BV symptoms in Perpignan in November–December 2000. White box with − indicates absence of BV or the viruses and gray box with + indicates presence of BV or the viruses detected.

Thus, no clear correlations emerged. These results, obtained only at one point in time, led us to analyze the dynamics of symptom development and virus presence over a period of weeks.

Time-course analyses

Results from the field near Perpignan are shown in Figure 2. In the first survey (November 29), among the 20 plants showing BV, 19 were positive for both MiLV and LBVV, and one plant (No. 8) was positive only for MiLV. Of the 20 symptomless plants, 10 were negative for both viruses, 10 were positive for LBVV, and five of these also contained MiLV. When the plants were studied on December 20, all plants earlier showing symptoms still showed them, and ELISA results were also the same, plant No. 8 remaining positive for MiLV only. Of the 20 plants that were symptomless in the first survey, the five plants originally positive for both viruses now showed symptoms and remained positive for both viruses. In addition, one more plant, originally positive only for LBVV, developed symptoms and was now positive for both viruses. All 14 plants which remained negative for MiLV remained symptomless; they comprised nine plants free of both viruses and five in which only LBVV was present. All these plants remained symptomless up to the end of the survey (January 5).

In Italy, of the 20 plants that showed symptoms in the first survey (April 4), five were positive for MiLV alone or the mixture, 12 contained LBVV alone or the mixture (Figure 3), with five apparently not containing either virus. Two weeks later, all but two of the plants

	April 4			April 18			May 9
	ELISA			ELISA			
Plant No.	MiLV	LBVV	Big-vein	MiLV	LBVV	Big-vein	Big-vein
1	+	-	+	+	+	+	+
2	-	-	+	+	+	+	+
3	-	+	+	+	+	+	+
4	-	+	+	+	+	+	+
5	+	+	+	+	+	+	+
6	-	+	+	+	+	+	+
7	+	-	+	+	+	+	+
8	+	+	+	+	+	+	+
9	-	+	+	+	+	+	+
10	-	-	+	+	+	+	+
11	-	-	+	-	+	+	+
12	-	+	+	+	+	+	+
13	-	+	+	+	+	+	+
14	+	-	+	+	+	+	+
15	-	+	+	+	+	+	+
16	-	+	+	+	+	+	+
17	-	+	+	+	+	+	+
18	-	-	+	+	+	+	+
19	-	+	+	-	+	+	+
20	-	-	+	+	+	+	+
21	+	+	-	+	+	+	+
22	-	+	-	-	+	-	+
23	-	-	-	+	+	-	+
24	-	-	-	+	+	-	+
25	-	-	-	+	+	-	+
26	-	+	-	-	+	-	+
27	+	+	-	+	+	+	+
28	-	+	-	+	+	-	+
29	-	+	-	+	+	-	+
30	-	-	-	+	+	-	+
31	-	+	-	+	+	-	+
32	-	-	-	+	+	-	+
33	-	+	-	+	+	-	+
34	-	-	-	+	+	-	+
35	-	+	-	+	+	-	+
36	-	-	-	+	+	-	+
37	+	+	-	+	+	+	+
38	-	-	-	+	+	-	+
39	-	+	-	+	+	+	+
40	-	+	-	+	+	-	+

Figure 3. Detection of MiLV and LBVV and development of BV symptoms in Torino in April 2001. White box with − indicates absence of BV or the viruses and gray box with + indicates presence of BV or the viruses detected.

carried both viruses. Of the 20 initially symptomless plants in Italy, three initially contained MiLV, alone or mixed, whereas 12 contained LBVV, alone or mixed. Two weeks later, 18 of the plants contained MiLV and all contained LBVV. At this time, only four plants (all positive for both viruses) showed BV, but on May 9, all 20 plants showed symptoms.

Discussion

The antiserum prepared against MiLV was specific, and suitable for ELISA. The antiserum to LBVV (Vetten et al., 1987) did not react with MiLV, though it was prepared from field lettuce that could have contained MiLV as well as LBVV. The second LBVV antiserum used, against the BR8 strain, never had any possible contact with MiLV antigens. The antisera did not cross-react and provided a reliable tool for the work that followed. It appears to be merely coincidence that in Western blots both viruses gave a band at a similar level representing 48 kDa; it is already reported (Roggero et al., 2000; Mayo, 2000) that each virus has a coat protein of the same apparent size.

Confirmation of the findings of Roggero et al. (2000), that MiLV as well as LBVV occur in lettuce crops has been briefly noted (Verbeek et al., 2001). However, the present paper appears to be the first report of extensive field surveys of lettuce crops in relation to BV, made with the realization that MiLV as well as LBVV could be present. In general, both viruses occurred at high frequency and they often occurred together. This may explain why the more easily detected LBVV has always been considered, it now appears erroneously, to be the causal agent of BV. As would be expected from the role of MiLV as the causal agent (Lot et al., 2002), plants with BV were frequently infected by MiLV (alone or in combination with LBVV). The surveys and time-course analysis done in France in November–January in plastic tunnels clearly supported the thesis that MiLV alone (or mixed with LBVV) causes BV, and that LBVV alone does not. There was no evidence of synergism between the two viruses in mixed infections.

The surveys and time-course analysis done in Italy, in open fields, did not give such clear results. For example, in the analysis of April 18, two plants out of 24 were still MiLV-negative but LBVV-positive by ELISA, and were scored as showing BV symptoms. It is possible that in these discrepant cases ELISA was not sensitive enough to detect MiLV at low concentrations or that the symptoms scored as BV were caused by another agent. A last possibility is that some field strains of LBVV can indeed cause BV; this seems unlikely as three separate isolates of LBVV, one French and two Italian, both from the same field as analyzed in the April experiments above, failed to cause BV, as reported by Lot et al. (2002).

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