Lettuce Ring Necrosis, a Viruslike Disease of Lettuce: Evidence for Transmission by *Olpidium brassicae*

R. N. Campbell, Department of Plant Pathology, University of California, Davis, CA 95616, and **H. Lot,** INRA, Station de Pathologie Végétale, B. P. 94, 84143 Montfavet Cedex, France

ABSTRACT

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Lettuce ring necrosis (LRN) caused severe symptoms on butterhead and crisphead types of lettuce but only mild symptoms on iceberg or cos types. Single sporangial isolates of *Olpidium brassicae* were prepared from three sites in France to prove the vector role of the fungus and to attempt the separation of the LRN agent (LRNA) and lettuce big vein virus (LBVV). A vector role for *O. brassicae* was demonstrated, but few isolates transmitted either agent, apparently because they were lost during one to two generation transfers of the fungus. Isolates transmitted LRNA and LBVV together or LBVV alone, but there was no clear evidence for transmission of LRNA alone. The LRNA was carried within the resting spores of the vector. Isolates of *O. brassicae* from two fields in the Salinas Valley of California carried LBVV and LRNA. This is the first report of LRNA in North America.

Lettuce ring necrosis disease (LRN), first described as kringnecrosis in the Netherlands and Belgium (9,18), has been observed in France, where it was called maladie des taches orangées (13). In southern France, the disease is observed primarily on the first winter crop of lettuce (*Lactuca sativa* L.) in protected culture. This crop is planted in September in plastic-covered tunnels or glasshouses and harvested December through January. The disease symptoms develop when the crop approaches maturity, the day length is short, and both light intensity and temperature are low. Symptoms are variable depending on the lettuce type and environmental conditions (H. Lot, *unpublished*).

Although LRN is soilborne and has been associated with infection by *Olpidium brassicae* (Woronin) P.A. Dang. (18), the fungus is a common inhabitant of lettuce roots, and better evidence of the vector role is needed (2). Likewise, the putative LRN causal agent (LRNA) has not been characterized. Mechanical transmission of the LRNA to lettuce and several other species failed (18). *O. brassicae* has been established as a vector of lettuce big vein virus (LBVV) (3,7), which it carries internally in resting spores (1). The LBVV was mechanically transmitted with difficulty to a few species but not to lettuce (8). The LBVV has labile, rod-shaped particles about 320 to 350 × 18 nm (10,11,19). Three other disease agents have been associated with *O. brassicae,* but they have not been characterized. A necrotic disease of cos or romaine lettuce in California caused necrosis of the older leaves and eventually death of the plant (14). The pepper yellow vein agent, first described from pepper (*Capsicum annuum* L.) (6), may have a wider host range that includes lettuce (15). The causal agent of freesia leaf necrosis was soilborne (17).

The objectives of this study were to establish a soil transmission protocol for LRN, to prove the vector role of *O. brassicae*, and to attempt the separation of LBVV and LRNA by isolating single-sporangial cultures of the vector that might carry none, one, or both agents. A brief report has been published (4).

Corresponding author: R. N. Campbell
E-mail: rncampbell@ucdavis.edu

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MATERIALS AND METHODS

Samples consisting of soil and fine roots surrounding symptomatic plants in protected cultures were collected in southeastern France. Sample A was collected from lettuce plants with typical lettuce big vein disease (LBV) symptoms in a glasshouse. Sample G was collected from plants with LRN symptoms in a plastic tunnel in which other lettuces had LBV symptoms. Sample T was collected from the few plants with LRN symptoms in another tunnel on the same property. The soils were air-dried in open plastic bags and stored in a cold room at 8 to 10°C.

The air-dried samples that had been stored for 9 months to 3 years were used to demonstrate soil transmissibility of LRNA and to establish cultures of *O. brassicae*. Soil transmissibility was demonstrated by sowing seeds in a 1:4 (vol/vol) mixture of sample G and sterilized peat potting soil in peat cubes, and growing the plants to maturity. Bulk cultures of *O. brassicae* were isolated by mixing sand in small pots (100 ml volume) with either 5 to 10 g of infested soil or root fragments picked from the samples. Bait seedlings were grown in the pots for 3 weeks. Root washings were prepared and examined for zoospores of *O. brassicae*, which were transferred to healthy seedlings in sand culture. The bait seedlings were transplanted to assay for LBVV and LRNA. The bulk cultures of *O. brassicae* were maintained on lettuce seedlings in a growth chamber at 18°C, and single sporangial cultures, identified by the SS prefix, were isolated using standard techniques (2). The length of a vegetative generation was prolonged by withholding water to prevent zoospore release (2,5). Single sporangial isolates were assayed for LBVV and LRNA 3 weeks after isolation and a year later when they were recovered from resting sporangia in dry roots.

The European crisphead (batavia) lettuce cv. Danilla was used for all culture work with *O. brassicae*. Additional lettuce cultivars used were butterhead types Melina and Ramona, European crisphead type Copelia, cos type Parris Island, and iceberg types Salinas, Pacific, and Climax. Copelia and Ramona were used because some growers consider them less susceptible to LRN. Pacific was reported to be resistant to LBV (16). Pepper cv. Yolo Wonder was included in some trials.

The seedlings being assayed for LBVV and LRNA were either the bait seedlings sown in infested samples or bait seedlings grown in sand infested with samples for three weeks and transplanted into sterilized potting mix in individual pots (14 cm diameter). The pots were kept in a glasshouse that was heated only to maintain minimum night temperatures of 4 to 6°C. The pots were placed on metal frames on soilless benches to prevent capillary uptake of water on the bench. Symptom expression began about 40 days after seeds

were sown in infested samples. A more rapid assay was used for *O. brassicae* cultures. The zoospores in a root washing were inoculated into 10 to 20 seedlings 4 to 8 days old in sand in a small pot. These seedlings were checked for *O. brassicae* infection about 10 days after inoculation and transplanted into pots as described above. Symptoms began to develop between 20 and 30 days after inoculation. All LRN trials were done during the period from mid-September until January (once until late March) to provide temperature and light conditions that favored severe LRN symptom expression. Such conditions were also satisfactory for LBV symptom expression. One assay was done in a growth chamber at Davis. The chamber was operated at 18°C for 32 days after the assay plants were inoculated and then at 16°C during the light and 14°C during the dark.

Three LBVV-transmitting isolates of *O. brassicae* (SS61, SS72, and SS73) were recovered from dry roots. These isolates had been prepared previously in California using lettuce cv. Climax and soil from two fields known to be infested with LBVV in the Salinas Valley.

Roots containing abundant resting spores of *O. brassicae* were used for acid treatment. The roots were air-dried for 1 day and pulverized in a mortar with a pestle. Half of each sample was treated with 1 N HCl for 2 h and the other half with water (2). The fungus was recovered and assayed for LBVV and LRNA by inoculation to Climax and Danilla indicator plants.

RESULTS

Soil transmission, symptomatology, comparison of cultivars. In the first soil transmission trial, sample G contained infectious LRNA and the vector after storage for 9 months. Cultivars Danilla and Melina were both susceptible; 44 and 37 of 50 plants, respectively, developed symptoms by 70 days after planting. Noninfested controls remained symptomless. Pepper plants had no symptoms of pepper yellow vein or LRN. On butterhead lettuce, angular or circular yellow spots appeared first, giving an irregular blotchy appearance to the basal and intermediate leaves (Fig. 1A and E). Large orange spots that became necrotic appeared on the lower surface of these leaves (Fig. 1F). A few necrotic rings appeared along the midribs. The first symptoms on crisphead lettuce were visible on the upper surface of the basal leaves as necrotic rings and yellow mottling (Fig. 1C) and on the lower surface as orange, irregular spots that became necrotic (Fig. 1B and D). These symptoms developed on the lamina of leaves of intermediate age but never on the youngest leaves within the head.

The cvs. Copelia, Ramona, Pacific, and Salinas were added in the second test 1 year later. Symptoms of LRN were pronounced on the butterhead and crisphead cultivars, with 90 to 100% of the plants developing symptoms by harvest time. About 10% of the iceberg cvs. Pacific and Salinas developed mild LRN symptoms consisting of a few necrotic rings near the midrib of a few leaves. Noninoculated plants of all cultivars remained symptomless.

Unifungal cultures of *O. brassicae* and transmission of LRNA. Bulk cultures of *O. brassicae* were readily obtained from each of the infested samples. Both LBVV and LRNA were present in samples A and G, as well as in SS61 from California (Table 1). Symptoms of LBVV started to appear 39 days after the plants had been sown and symptoms of LRNA after 42 days. Culture T did not produce symptoms; presumably the titers were low because symptoms developed on a few plants in later transfers. The noninfested controls were free of *O. brassicae*, LBVV, and LRNA. When bulk cultures G and T and SS61 were increased and reassayed on four cultivars, culture G produced mostly LRN symptoms, whereas SS61 produced mostly LBV symptoms (Table 2). The first symptoms of LBV and LRN were noted 31 days after inoculation. Big vein symptoms developed earlier and were expressed more distinctly on cv. Climax than on Danilla and Melina; otherwise, all three cultivars were good indicators for LBVV. Cultivars Danilla and Melina were better indicators for LRN than were Climax and Parris Island. Thus, Climax and Danilla were selected as indicator cultivars for LBVV and LRNA, respectively.

Single sporangial isolates of *O. brassicae* were made, with special emphasis on culture G, which seemed to have the highest titer of LRNA. The isolates were assayed for LBVV and LRNA 3 weeks after they were made and again the following year after recovery from dry root samples. None of 17 single sporangial isolates from culture G transmitted LBVV or LRNA in either assay. One of four single sporangial isolates from culture A (SS141) transmitted both LBVV and LRNA. In the second year, five more single sporangial isolates were prepared from SS141. Two isolates transmitted both LBVV and LRNA (SS155 and SS156), and three did not transmit either agent. Another isolate (SS138) from culture A produced unusual necrotic lesions on the upper surface of some leaves of one plant of Danilla and orange lesions on the lower surface. Isolate SS138 was rated as questionable for LRNA because only one plant had symptoms in assays done over two seasons, and these symptoms were not as severe as with other isolates. None of three isolates from culture T had LBVV or LRNA, although two isolates were not recovered for retesting in the second year. The three isolates from California were included in the assays in the second year, and all three isolates transmitted LBVV as they had done when they were originally isolated. Two isolates, SS61 and SS73, also transmitted LRNA.

To confirm the existence of LRNA in California, isolate SS61 was recovered from dry roots and assayed in a growth chamber at Davis using three lettuce cultivars. The first LBV was observed on Climax seedlings 23 days after inoculation and on Danilla and Parris Island at 35 days postinoculation. Symptoms of LRN were seen only on Danilla. On the 48th day, six of eight plants had LRN and of the six, five also had LBV symptoms. By this time nine of nine Climax plants had LBV but no LRN.

Acid treatment trials. Four samples of roots infected by SS141 and one infected with culture G were used for an acid treatment trial. *O. brassicae* survived in all treated and control replicates and continued to transmit both LBVV and LRNA, except in the single acid-treated replicate of culture G (Table 3). The noninoculated controls were free from *O. brassicae* both at the time of fungus recovery and at transplantation, and were negative for LBV and LRN. Two additional replicates of indicator plants were inoculated with SS61 and maintained with this trial to confirm that SS61 carried LRNA. Nine and six of 10 indicator plants developed LBV and LRN, respectively.

DISCUSSION

We have demonstrated that unifungal (single sporangial) isolates of *O. brassicae* transmit LRNA; therefore, *O. brassicae* is the vector of LRNA. The LRNA is carried internally in the resting spores of the fungus, where it is protected from acid treatment. LRNA was found also in isolates of *O. brassicae* from California. The latter isolates had been made and stored in dry roots before transfer of research materials from California to France.

LRNA is a disease of the crisphead and butterhead lettuces, the main types cultivated in protected culture in France and other parts of Europe. The crisphead cv. Danilla did not differ in susceptibility from other crisphead and butterhead cultivars. Thus, the growers' observations that it was more susceptible (H. Lot, *unpublished*) probably reflected the popularity of this cultivar, which comprised over 50% of the acreage of winter crisphead lettuce.

The combination of environmental conditions that favor symptom expression in Europe in winter has not been established. Low light intensity, short day length, and minimum temperatures near freezing seem to be involved because of the seasonal pattern of disease development. LRN probably has not been recognized in California because of host plant resistance and environmental effects. The iceberg cultivars grown in California appear to be highly tolerant with limited

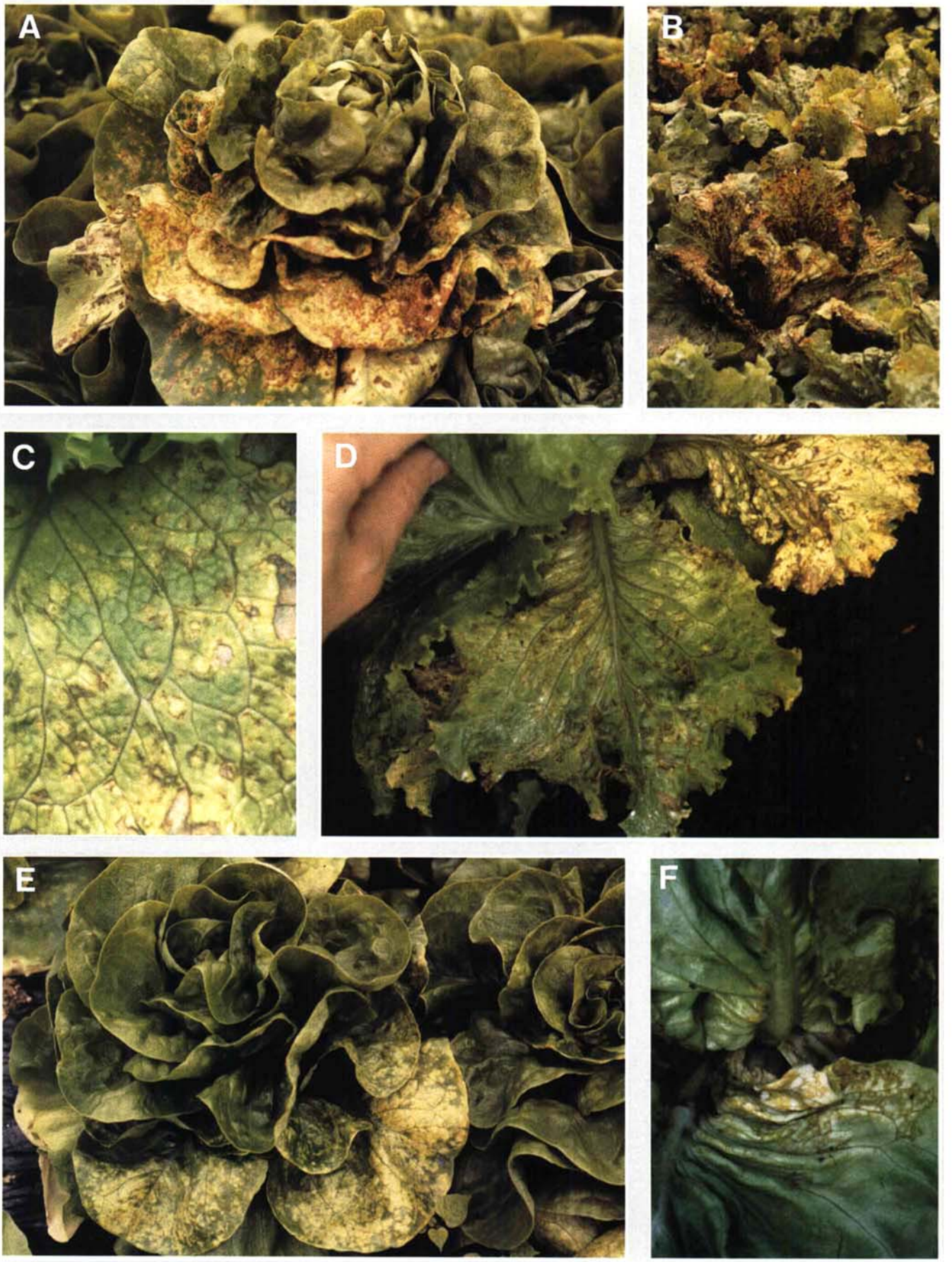

Fig. 1. Symptoms of lettuce ring necrosis on plants grown in infested soil. Butterhead lettuce in A, E, and F; crisphead lettuce in B to D; all plants from experimental plantings except A and B, which are from commercial crops. **(A)** Yellow spotting and necrotic rings; **(B)** advanced necrosis on intermediate-young leaves; **(C)** primary symptoms of yellow mottling and brown rings on a basal leaf; **(D)** advanced yellowing and orange spotting; **(E)** primary symptoms of yellow mottling on basal and intermediate leaves; **(F)** orange rings and spotting at base of intermediate leaves.

symptom expression, even when young seedlings are inoculated with large numbers of zoospores. Winter lettuce grown in the deserts of southern California is exposed to freezing temperatures, but the longer day and greater light intensity compared to Europe at the same season may preclude symptom expression.

Plants showing only LRN symptoms have been noted in our bulk cultures of *O. brassicae* and in growers' crops. Yet when transfers are made, both LRN and LBV symptoms develop. We assume this pattern results from the relative titers of the agents in the inoculum, the method of inoculation, or the environment being too cool for optimum expression of LBVV (20). We included Parris Island cos and pepper in our trials but did not obtain the disease described on cos (14) or pepper yellow vein (15).

The failure of all 17 single sporangial isolates from culture G to transmit either LRNA or LBVV was unexpected, because about 50% of such isolates generally transmit LBVV (12; R. N. Campbell, *unpublished*). The only difference from the usual procedure was in the handling of this culture prior to extraction of single sporangia. Ordinarily, a stock culture grown for 2 to 6 weeks on a host plant is inoculated to seedlings from which the first generation of single sporangia are isolated (2,12). In the present work, culture G was inoculated to and increased on seedlings for two vegetative generations. The fungus was then inoculated to pots of seedlings from which seven single sporangia were isolated or which were used to produce zoospores to inoculate additional pots from which 10 single sporangia were isolated. Apparently the titers of LBVV and LRNA were greatly reduced or lost when the fungus was passaged for only one or two generations on healthy plants. This hypothesis is supported by assays done the following year for the agents in roots saved from additional pots that were inoculated, but not used for the isolations, and later dried. The fungus recovered from roots corresponding to the two-generation increase hosts transmitted LRNA but not LBVV. The fungus from roots corresponding to the second set of 10 single sporangial isolates transmitted neither LBVV nor LRNA.

The mechanism of in vivo acquisition of virus by fungal vectors has had little study. The loss of LBVV and LRNA during one- or two-generation transfers of the fungus could occur if a large proportion of the agent carried into the host by the zoospore protoplast were released into the host cytoplasm before the thallus formed a cell wall and if acquisition of virus was likewise restricted to early stages of growth of the fungus in a virus-infected host cell.

The identity of the LRNA remains elusive, although it probably is a virus. The symptomatology of LRN differs markedly from that of LBV, but they have similar incubation periods. LBV begins as vein banding on young, expanding leaves that continue to show this symptom as they grow. LBVV may cause a more upright growth habit of the leaves of iceberg lettuce, but it does not induce yellowing and necrosis as does LRN. LRN starts on the older leaves and later involves intermediate leaves. Both LBVV and LRNA have the same vector. We have obtained vector isolates that transmit LBVV alone or that transmit LBVV and LRNA, but none that clearly transmit just LRNA. It is possible that such isolates might be found if additional isolates were made. Thus, LRNA could fit several models: it could be a symptomatological strain of LBVV, a satellite virus or satellite nucleic acid of LBVV, or a distinct virus. Whatever the nature of LRNA, its widespread occurrence means that it could confound research done with supposedly pure cultures of LBVV. Labile, rod-shaped particles have been observed in dip preparations from plants with LRN symptoms (H. Lot and B. Delecolle, *unpublished*). These particles were decorated with the LBVV antiserum of Vetten et al. (19) diluted up to 1/1,026. These results support the first hypothesis, and further work is in progress to confirm the results.

Table 1. Number of bait seedlings of lettuce cv. Danilla developing symptoms of lettuce big vein (LBV) or lettuce ring necrosis (LRN) after growing in an unheated greenhouse for 9 weeks

	Number of plants[a]			
Soil source	**LBV**	**LBV+LRN**	**LRN**	**Symptomless**
G	0	2	17	1
A	4	5	1	10
T	0	0	0	20
SS61	4	4	0	8
Control	0	0	0	20

[a] Ten bait seedlings from each of two replicate pots were transplanted into individual pots after assaying for *Olpidium brassicae*, which was found in all except the control treatment.

Table 2. Comparison of four lettuce cultivars for expression of lettuce big vein (LBV) and lettuce ring necrosis (LRN) in three cultures of *Olpidium brassicae*

		Number of plants[a]			
Culture	**Cultivar**	**LBV**	**LBV+LRN**	**LRN**	**Symptomless**
G	Climax	1	0	1	18
	Parris Island	0	0	3	17
	Danilla	0	0	14	6
	Melina	0	0	10	10
T	3 cultivars[b]	0	0	0	60
	Melina	0	0	1	19
SS61	Climax	16	0	0	4
	Parris Island	10	1	0	9
	Danilla	15	0	0	5
	Melina	16	0	0	4
Control	4 cultivars[c]	0	0	0	80

[a] Twenty plants for each cultivar and fungus source consisted of 10 plants grown in a glasshouse and 10 grown in a plastic-covered tunnel.
[b] Climax, Parris Island, Danilla (combined).
[c] Three cultivars above plus Melina.

Table 3. Transmission of lettuce big vein virus (LBVV) and lettuce ring necrosis agent (LRNA) after acid treatment of resting spores of *Olpidium brassicae*

		Results		
Culture	**Treatment**	***O. brassicae*[a]**	**LBV[b]**	**LRN[c]**
SS141	Acid[d]	4/4	18/20	16/20
SS141	Control	4/4	20/20	15/20
G (bulk)[e]	Acid	1/1	0/5	0/5
G (bulk)	Control	1/1	4/5	2/5
No fungus	None	0/2	0/10	0/10

[a] No. replicates positive for *O. brassicae*/no. tested.
[b] No. Climax indicator plants with LBV symptoms/no. tested (five plants/replicate).
[c] No. Danilla indicator plants with LRN symptoms/no. tested (five plants/replicate).
[d] Resting spores immersed in 1 N HCl for 2 h.
[e] A culture propagated by mass transfer of zoospores prior to isolation of unifungal cultures by means of single sporangia.

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