Lettuce Big-Vein Virus Transmission by Single-Sporangium Isolates of Olpidium brassicae

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ABSTRACT

Of 18 single-sporangium isolates from a mass culture of *Olpidium brassicae* that transmitted lettuce big-vein virus (BVV), only 8 were viruliferous. Each of the 10 nonviruliferous isolates was capable of acquiring BVV in vivo and transmitting it. Of 13 additional single-sporangium isolates obtained from a viruliferous single-sporangium isolate, 12 transmitted BVV and the other isolate could acquire BVV in vivo. The over-all results suggest that all *Olpidium* isolates capable of multiplication in lettuce can acquire and transmit BVV. All attempts to mechanically transmit and characterize BVV failed; thus, the nature of BVV is still uncertain. Phytopathology 60:1630-1634.

Several studies have shown that *Olpidium brassicae* (Wor). Dang. (hereafter *Olpidium*) functions as the vector of an infectious, graft-transmissible agent that causes big-vein of lettuce, referred to herein as the lettuce big-vein virus (BVV) (4, 5, 18). Most previous studies have been done with mass cultures of *Olpidium* maintained in lettuce roots. The conclusions are therefore subject to reservations because of lack of pure, or axenic, cultures, and from possible genetic diversity of the fungus populations. Sahtiyanci (13) designated the BVV-transmitting fungus as *Pleotrachelus virulentus* Sahtiyanci and described its life cycle in detail. She isolated eight single-sporangium lines and reported that a zoospore suspension from one line transmitted big-vein. It was not clear, however, whether all lines were tested and only one transmitted the disease or whether only one line was tested. Furthermore, because of inadequacy of methods utilized, there is reason to question whether each line was actually derived from a single sporangium. BVV has been transmitted by grafting and by *Olpidium* zoospores, but despite much effort the nature of BVV has not been elucidated (4, 5, 18).

The present paper presents data on frequency of BVV transmission by single-sporangium isolates, and evidence that nonviruliferous single-sporangium isolates capable of multiplying in lettuce also were capable of acquiring and transmitting BVV. Also described are unsuccessful attempts to mechanically transmit, characterize, or visualize BVV in in vitro preparations and in infected tissues. Some of the data have been presented in abstract (12).

MATERIALS AND METHODS.—To provide material for isolation of single sporangia, lettuce (*Lactuca sativa* L. 'Climax') seeds were sown in 30-ml plastic beakers filled with pasteurized quartz sand (3). Approximately 4 days later, the seedlings were inoculated with a root washing containing *Olpidium* zoospores as well as rhizosphere microbes from the source plant (3). After incubation at 16 ± 2 C for 3-4 days, most sporangia were mature and ready to discharge zoospores (8). The roots were rapidly washed free of sand with running tap water and placed in 20 ml of chilled (2-5 C) 0.5 M glycine-NaOH buffer (pH 7.6) in a small (360-ml capacity), chilled, stainless-steel blender jar. After blending the contents for 20-30 sec, the homogenate was poured onto a 250-mesh soil sieve. Both blender jar and sieve were sterilized before use by immersion in 2% formaldehyde for at least 0.5 hr. The filtrate was collected in a beaker kept in an ice bath, and drops of filtrate were streaked over the surface of chilled, sterile 2% water agar in a petri plate. The agar surface was examined and a single, apparently mature, sporangium well isolated from other sporangia (Fig. 1) was selected and transferred to a small drop of chilled sterile tap water on a chilled slide by removing a plug of agar (about 1-mm diam) on which the sporangium was located, and dipping the agar plug into the water drop. This drop was carefully examined with a phase-contrast microscope to detect other sporangia or zoospores. Usually none was present and, if not, the sporangium was washed into a 30-ml plastic beaker containing 4-day-old lettuce seedlings grown as described. With this method, it was possible to make about 15 single sporangium isolations in 4 hr. Usually only one or two sporangia were isolated from each petri plate because rising temp permitted zoospore discharge. After incubation at 16 C for 3-4 weeks, a root washing (4) was made from the plants in each beaker and checked microscopically for zoospores. In various trials, 40 to 90% of the inoculated units had infected plants. Each of these single-sporangium isolates, hereafter termed *isolates*, was transferred to healthy lettuce seedlings for increase.

During the preliminary experimentation as the technique was being developed, the agar block was also transferred to a second water drop that was washed into a beaker of seedlings to check for contamination. At other times, the sporangium was removed from one water drop with a micropipette and placed into another drop to discharge zoospores while the first drop was inoculated to a beaker of seedlings as a control. In addition, an equal number of noninoculated units was placed among the inoculated in every group of isolations. None of these controls was infected by *Olpidium*. Although in early trials the technique succeeded when
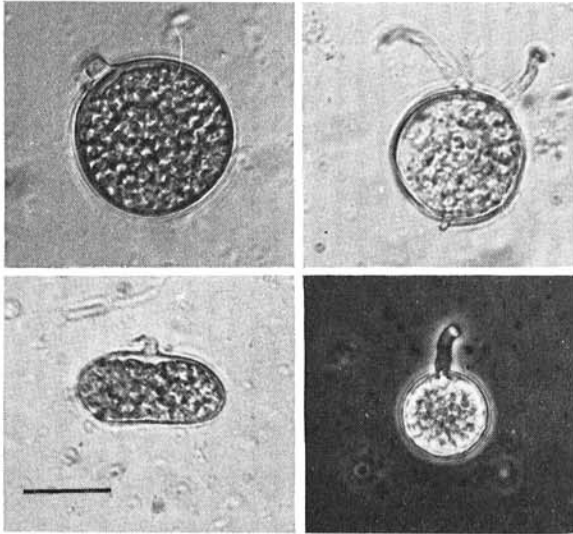
Fig. 1. Single sporangia of *Olpidium brassicae* extracted from lettuce root cells and streaked on agar surface. Bar represents 20 μ.

sporangia were extracted in chilled 0.05 M glycine buffer or tap water, zoospore release occurred so rapidly that only one or two sporangia could be isolated from each filtered homogenate before zoospores appeared. Later it was found that the combination of chilling and 0.5 M glycine buffer inhibited zoospore release for 2 hr, the longest time tested, and the technique described at the beginning of this section became standard.

In the present study, isolates were obtained from three mass cultures. One was a BVV-transmitting culture from lettuce (3), another was a tomato *Olpidium* free of BVV at the time of isolation on lettuce (15), and the third was a BVV-free culture derived from serial transfer of the BVV culture on sugarbeet roots (1). The mass cultures, stored as resting spores in dry roots for 1 year before this study was begun, were inoculated to lettuce plants. One month later a root washing was made and the resulting zoospore suspensions were transferred to fresh seedlings that were kept in a growth chamber at 16 C for 1 month before the isolations were begun.

Assays for the presence of BVV in the isolates were done by transferring 5×10^5 zoospores to lettuce seedlings in a 30-ml beaker. These and an equal number of noninoculated controls were incubated for 2-3 weeks at 16 C, with care to prevent contamination between beakers. Ten to 15 inoculated seedlings or corresponding noninoculated controls were transplanted individually or in pairs to pots with a pasteurized greenhouse potting mixture. These were incubated for a further 5 weeks either during the winter in a cool greenhouse where air temp at night was maintained at 18 C and the day temp was generally below 24 C, or during the summer in an air-conditioned chamber with a constant temp of 18 C. The leaf symptoms of BVV usually appeared within 1 to 2 weeks after transplant-

ing. Although an isolate would be recorded as positive if only one assay plant developed symptoms, more than 75% usually developed symptoms. Symptoms of BVV never showed on the plants of the noninoculated controls that were placed in alternate rows between the inoculated plants. All isolates formed resting spores, and a stock culture of each isolate was maintained as resting spores in dried roots.

The acquisition of BVV was done by the in vivo technique (5). Each BVV-free isolate was increased in lettuce seedlings. A zoospore suspension was divided into two equal portions, one of which was inoculated to a graft-inoculated BVV plant, and the other to a comparable healthy control plant. After approximately 2 weeks, zoospores from each plant were inoculated to small seedlings in a beaker and assayed for BVV as described above. An additional control consisting of noninoculated lettuce seedlings was maintained in the same manner in each trial.

RESULTS.—*Presence of BVV in single-sporangium isolates.*—Of eighteen isolates obtained from the BVV-transmitting mass culture, only six were viruliferous in the first assay for BVV (Fig. 2). The apparently nonviruliferous isolates were retested, and two additional ones were found to be viruliferous. When tested for the ability to acquire BVV in vivo, each of the 10 nonviruliferous isolates acquired and transmitted BVV, although two isolates were negative in the first trial and had to be retested. The controls in all experiments remained free of BVV symptoms.

Some 14 weeks after the first isolations were made, 13 additional isolates were obtained from one of the viruliferous isolates and 12 were viruliferous (Fig. 2). The one isolate that did not transmit BVV apparently failed to acquire it in the first acquisition test but did so in a second test. The controls remained free of BVV symptoms in all cases.

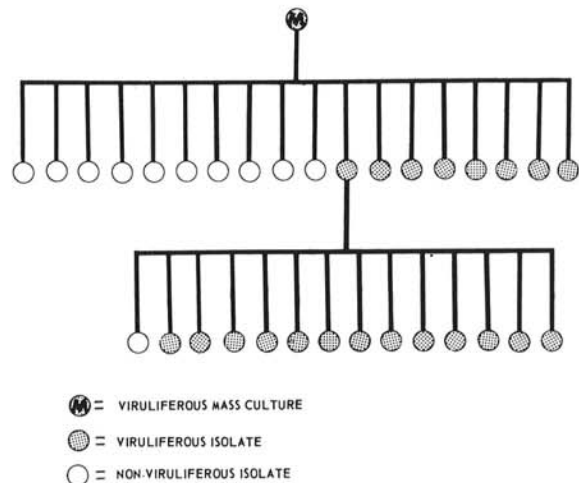Five additional nonviruliferous single-sporangium isolates, one from the tomato culture and four from

Fig. 2. Transmission of lettuce big-vein virus (BVV) by single-sporangium isolates derived from a BVV-transmitting mass culture of *Olpidium brassicae.*

the BVV-free mass culture, were each capable of acquiring and transmitting BVV in trials in which the controls remained free of BVV symptoms.

Attempts to characterize BVV.—Attempts to mechanically transmit BVV by a number of methods have been uniformly unsuccessful. The following summary indicates the types of experiments that have been done, but because results are negative details of each experiment are omitted. The host range inoculated generally included lettuce, *Vigna sinensis* (Stickm.) Savi ex Hassk.; *Cucumis sativus* L.; *Phaseolus vulgaris* L.; *P. aureus* Roxb.; *Datura stramonium* L.; *Nicotiana tabacum* L. 'Havana'; *Chenopodium amaranticolor* Coste & Reyn.; *Gomphrena globosa* L.; and occasionally *Emilia sagittata* L. and *Montia perfoliata* (Donn.) Howell. The host range was kept for 4 weeks or longer under conditions favorable for BVV symptom expression by lettuce seedlings inoculated with viruliferous *Olpidium*. Generally, leaves with BVV symptoms were triturated and the extract was applied to Celite-dusted leaves of host plants. These trials included the following solvents or methods of preparation: extract in 0.01 M phosphate buffer (pH 7.6) alone or with 0.001 M 1-phenylthiosemicarbazide inoculated to leaves of host range plants (10); extract in liquid nitrogen-10% sucrose inoculated to leaves of host range plants (6); extract in 0.005 M phosphate buffer with 0.2% mercaptoacetic acid precipitated with two volumes of ethanol and the precipitate dissolved in 0.01 M phosphate buffer. Some of this solution was mixed in vitro with BVV-free zoospores of *Olpidium* that were inoculated to lettuce seedlings and the remainder was made up to 10% sucrose and either inoculated to the leaves of the host range plants or to the roots of lettuce seedlings by excision of the root tips in the solution and later transplanting into sand in pots to observe symptom expression; extract in 10% sucrose inoculated to the roots of lettuce seedlings by excising their root tips excised in solution; extract in 0.5 M phosphate buffer with water-saturated phenol, sodium dodecyl-sulfate, and Mg bentonite [modified from the RNA extraction method of Gillaspie & Bancroft (9) and prompted by the observations of Diener & Raymer (7) with potato spindle tuber virus nucleic acid], precipitated with ethanol and the resuspended precipitate inoculated to lettuce leaves; the razor-slash technique developed for citrus exocortis virus (14). None of these experiments resulted in transmission of BVV to healthy lettuce or in development of abnormal symptoms on other plants of the host range.

Attempts were made to conc extracts from leaves of healthy (*Olpidium*-free) and diseased plants and to compare them for anomalous components that might represent BVV or associated products of infection. In these attempts, crude sap extracts were prepared by blending 1.5 ml of 0.05 M phosphate buffer (pH 7.6) with 0.1 M ascorbic acid/g of leaf. In one trial, extracts from 73-g samples of leaves were centrifuged at 26,000 rpm for 15 min (Spinco 50.1 rotor) and the pellets were resuspended, clarified, and placed on 10-40% sucrose density-gradient columns (centrifuged in Spinco 25.1 rotor at 22,500 rpm for 30 min). The columns were scanned at 254 nm in an ISCO density-gradient fractionator, but there were no differences between healthy and diseased extracts. Attempts to isolate "small" particles (sedimented at 45,000 rpm for 60 or 90 min in 50.1 rotor) yielded differences between extracts from healthy and from BVV-infected leaves. For example, in one trial, 90-g samples of leaves were extracted and subjected to two cycles of differential centrifugation. After the second high-speed centrifugation, the pellets were resuspended and ultraviolet absorption was compared. The healthy extract yielded a total of 14 OD units (A_{260}) with a broad plateau from 240 to 270 nm. The BVV extract yielded a solution with 128 OD units (A_{260}) and an absorption curve ($A_{max} = 258$ nm, $A_{min} = 238$ nm, max/min = 1.39). When the BVV extract (diluted to 40 OD units/ml) was compared with the healthy extract in a Spinco Model E analytical ultracentrifuge, there were no qualitative differences in the sedimentation patterns (determined by schlieren optics) of healthy and BVV extracts.

Since one characteristic of the mycoplasma-type diseases is that symptoms are suppressed while infected plants are treated with tetracycline antibiotics (11), several types of treatment were done with chlorotetracycline HCl (aureomycin) at 10 or 100 ppm to attempt suppression of BVV symptoms. These treatments included (i) washing sand from the roots of graft-inoculated plants with symptoms and placing their roots in solutions for 24 hr before repotting them; (ii) watering plants with the test solutions at 1, 7, and 14 days after inoculation with zoospores; or (iii) inoculating plants with zoospore suspensions containing 10 or 100 ppm plus watering the respective test solutions onto the plants as in (ii) above. There was no remission of symptoms or delay in symptom expression or reduction in the percentage of plants developing symptoms.

Attempts also were made to associate characteristic submicroscopic structures with BVV infection. The host tissues were prepared for electron microscopy by fixing, embedding, and staining as previously reported (16), except that leaves in the glutaraldehyde fixative were under vacuum for the first 10 min of the fixation period to obtain better penetration by the fixative. Zoospores were processed as previously described (17).

Five samples of leaves were fixed using veins and adjacent tissue from leaves with distinct symptoms, but only one sample of interveinal (apparently symptomless) tissue was examined. Special examination was made of phloem tissues. Roots inoculated by a viruliferous isolate were examined 3 days and 5 weeks after inoculation. In these specimens, the host cells as well as *Olpidium* sporangia were examined. Zoospores from an isolate that transmitted BVV were also examined. In no instance were particles of any of the known types of plant viruses (rod, polyhedral, bacilliform with membrane) observed in vascular or parenchyma tissue or in the vector zoospores or sporangia, and no mycoplasmalike bodies were observed in the

host phloem or in *Olpidium*. Chloroplasts in leaf tissues showing the typical BVV-veinbanding appeared to be fewer in number than in healthy controls, and had somewhat fewer cristae but were otherwise normal. The presence of chloroplasts in these cells helps to explain our observation that although symptoms may be severe on expanding, mature leaves, they become less distinct on these same leaves 1-2 months later. The chloroplasts apparently are not destroyed, but only inhibited in their development.

DISCUSSION.—The method used herein for obtaining single-sporangium isolates of *Olpidium* permits the routine testing of homogenous isolates. The nature of sexual reproduction in this fungus is uncertain; therefore the genetic homogeneity of the single-sporangium lines is uncertain. We assume that each vegetative sporangium arose from infection by a single zoospore, but it is yet to be determined whether zoospores that typically possess a single nucleus (17) are diploid or haploid or perhaps a mixture of each. When this and the method of resting spore formation are known, the genetic homogeneity of single-sporangium isolates can be assessed. Nevertheless, single-sporangium isolates provide a much more satisfactory type of inoculum for studying variability in morphology, in virus transmission, and in host range than do mass cultures.

The transmission of BVV by single-sporangium isolates provides additional evidence that *Olpidium* is the vector of BVV. The lack of BVV transmission in some single-sporangium isolates indicates that BVV is not uniformly distributed among all the *Olpidium* thalli, although the mass culture had been maintained for several years on BVV-infected lettuce. Isolates assayed as nonviruliferous either may have been totally free of BVV or have had such a small amount of BVV that it was lost during initial reproduction on lettuce; i.e., zoospore infection did not result in systemic BVV infection of roots and thus there was no acquisition by subsequent generations of *Olpidium*. The fact that two of the apparently nonviruliferous isolates were shown to be viruliferous in a second assay suggests that there was only a small amount of BVV in these isolates at the time of isolation. The absence of BVV in one of the second series of isolates suggests that among progeny from a viruliferous sporangium there were a few with no virus; i.e., BVV was not included in the cytoplasm of some zoospores as they were differentiated. These results also permit interpretation of earlier data showing loss of BVV transmission by *Olpidium* mass cultures when zoospore suspensions were diluted nearly to the dilution end point before transfer to lettuce plants, or when *Olpidium* was heated nearly to the thermal death point (5). It seems probable that the infectious zoospores or resting spores surviving the treatments were virus-free before the treatments started.

The acquisition experiments demonstrated that all virus-free isolates found in this study acquired BVV in vivo and transmitted it. This excludes the possibility that these isolates lacked this ability. Since the tomato culture also was able to acquire BVV, it seems likely that any isolate of *Olpidium* that can infect and multiply in lettuce roots can acquire and transmit BVV. Since only in vivo acquisition can be demonstrated, it is not possible to test *Olpidium* isolates that do not reproduce in a host of BVV. Only composites of the Chicorieae tribe that show veinbanding symptoms are at present regarded as hosts. Early reports of a wider host range may be due to BVV persistence in *Olpidium* rather than to host susceptibility to BVV (2). The isolates studied here were all capable of acquiring tobacco necrosis virus in vitro and transmitting it.

The techniques for electron-microscopic detection of viruses and of mycoplasmas have been developed in many laboratories and have given good results in our laboratory with tobacco necrosis and other viruses. Nevertheless, attempts to demonstrate anomalous chemical or morphological constituents in BVV plants or vectors have not been successful. Thus we can conclude nothing about the nature of the infectious agent. We shall continue to refer to it as lettuce big-vein virus (BVV) because it has the attributes of many other so-called viruses, graft transmissibility and vector acquisition and transmission. But the use of this virus name should continue to be regarded as provisional until the causal agent is characterized. The situation with BVV resembles that demonstrated for potato spindle tuber virus (7) or citrus exocortis virus (14), with the important exception that these naked-RNA viruses are mechanically transmissible. Progress in characterizing BVV seems unlikely unless a method for obtaining mechanical transmission can be developed.

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