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The Transmission of Satellite Viruses of Tobacco Necrosis Virus by *Olpidium brassicae*

By B. KASSANIS AND I. MACFARLANE
Rothamsted Experimental Station, Harpenden, Hertfordshire

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SUMMARY

None of four isolates of *Olpidium brassicae* (Wor.) Dang. (*Olpidia* 1, 2, 3 and 6) transmitted the original strain of satellite virus (SV 1). Two strains of satellite virus recently isolated (SV 2 and SV 3) were both transmitted, but SV 2 only by *Olpidium* 6 and SV 3 only by *Olpidium* 3. Hence different isolates of *Olpidium* seem to be specific vectors for different strains of satellite virus.

INTRODUCTION

Satellite virus multiplies only in the presence of tobacco necrosis virus (Kassanis & Nixon, 1961; Kassanis, 1962). In naturally infected plants, it occurs only together with tobacco necrosis virus, so the two viruses probably have the same vector. Teakle (1962) showed that tobacco necrosis virus is transmitted to the roots of host plants by the zoospores of *Olpidium brassicae* (Wor.) Dang. (henceforth called *Olpidium*). One of the virus inocula he used was an English culture of tobacco necrosis virus that contained the original strain of satellite virus, SV 1, and Teakle claimed that the fungus transmitted both viruses because when sap from the infected roots was inoculated on French bean and tobacco leaves it produced large and small lesions. However, although it is true that tobacco necrosis virus produces smaller lesions with satellite virus than alone (Kassanis, 1962), many other factors can also affect the size of lesion. The only conclusive way of showing that satellite virus is transmitted is to demonstrate the presence of the virus in infected roots serologically or by electron microscopy. Until now this has not been done. We confirmed Teakle's finding that tobacco necrosis virus can be transmitted by *Olpidium* but were unable to transmit SV 1 by either a Californian or two English isolates of the fungus. However, we have transmitted two recently isolated strains of satellite virus, SV 2 and SV 3. This paper describes the circumstances under which these new strains of satellite virus were isolated and their transmission by *Olpidium*.

METHODS

Plant culture. Transmission experiments were made with Mung bean (*Phaseolus aureus* Roxb.), French bean (*Phaseolus vulgaris* L., cultivar 'The Prince') and lettuce (*Lactuca sativa* L., cultivar 'Cheshunt 5 B') but mainly with tobacco (*Nicotiana tabacum* L., type 'White Burley', cultivar 'Judy's Pride'). Seedlings were raised and plants grown in sand culture as described by Kassanis & Macfarlane (1964).

Olpidium cultures. Olpidia 1, 2 and 3 were previously reported as were the methods of

culturing and handling the fungus (Kassanis & Macfarlane, 1964; Macfarlane, 1968). *Olpidium* 2 was kindly sent by Dr Teakle from California and *Olpidium* 6 was isolated during this work.

Viruses. The original strain of satellite virus SV 1 is the one used in all work so far published (Kassanis & Nixon, 1961; Kassanis, 1962). The new strains SV 2 and SV 3 were isolated during the present work. Of the different strains of tobacco necrosis virus described by Babos & Kassanis (1963), we used tobacco necrosis virus A or B to activate all three strains of satellite virus.

Experimental procedure. At first, Mung bean seedlings were used, grown either singly in vials, as in our previous work, or many plants in a crystallizing dish. The bean roots became so necrotic when infected that it was easier to obtain the volume of sap necessary for a serological test (about 2 ml.) from a larger plant, usually tobacco, grown in water culture or in sand.

For water cultures, tobacco seedlings were raised in sand, and when still very small (1 leaf) transferred to a tank of 1/2 strength Hoagland's solution and they were ready for use after 3 to 4 weeks. At inoculation plants were put in a crystallizing dish (50 ml. or 150 ml.) containing a suspension of *Olpidium* zoospores in 1/20 Hoagland's solution, pH 7, to which viruses had been added. The plants were left in the infection medium for at least 4 hr and then carefully transferred to glazed pots containing 2·5 l. 1/2 strength culture solution. Plants were supported by split rubber bungs in holes in bitumen-coated wooden lids. The solution was aerated intermittently and changed weekly.

When sand cultures were used, seedlings were transferred to sand in polyethylene pots (5·5 or 7·5 cm. rim diameter) or amber glass pots (8 cm. rim diameter). The smaller plastic pots were suspended in a small tank (Macfarlane, 1968) and irrigated like plants with fungus cultures, one tank being used per treatment. A simpler and more economical method but one that required closer supervision was merely to put the pots in saucers. Water and nutrient solution were alternately added to the saucers from separate beakers for each treatment. Inoculum was added by pipette close to the base of the plant stem. At inoculation enough liquid was put in the tank or saucer to keep the sand saturated and so give the zoospores full opportunity to infect. Wet conditions were maintained regularly throughout the experimental period.

Concentration of fungi and viruses in inocula. Plants to be grown in water culture were inoculated in a small volume of liquid to expose the roots to the greatest possible concentration of zoospores, which ranged from 5×10^5 to 10^7/ml. with *Olpidium* 3. The final solution contained infective sap at 1/50 or purified tobacco necrosis virus A at about 0·2 μg./ml. and 0·2 to 0·5 μg. satellite virus/ml.

In early sand-culture experiments, viruses and zoospores were added separately but in later ones viruses were mixed with zoospores and left for 15 min. before pipetting to the base of the plant. The final inocula contained sap, again diluted 1/50, or purified viruses at a concentration of 20 μg./ml.

Assay of viruses. The presence of tobacco necrosis virus in inoculated roots was established by infectivity and serological tests or electron microscopy, and that of satellite virus by serological tests and electron microscopy. Infectivity tests were made by inoculating sap extracted from roots on primary leaves of French bean dusted with carborundum. Serological tests were done with heat-clarified sap (10 min. at 50°) either by precipitation in narrow tubes or by precipitation-in-gel. For electron micro-

scopy, heat-clarified sap was mixed with sodium phosphotungstate and sprayed on to carbon films supported on seven-hole platinum mounts.

Control of contamination. Equipment that could not be sterilized by autoclaving was washed with detergent and then treated with a solution of sodium hypochlorite (3 g. Cl_2/l.), which experience showed was sufficient, for our purposes, to sterilize the surface of plastic pots. Great care was taken to avoid splashing when inoculating or watering. The precautions taken—for example, with pots in saucers—seemed to be sufficient to avoid the spread of aquatic fungi like *Olpidium* from one pot to another. Nevertheless, we had occasional contaminations with virus and, to avoid this, more stringent separation of treatments is necessary. Although, in general, virus transmission in the present experiments seemed less reliable than in our previous work with small seedlings, perhaps conditions occasionally favoured transmission from minute amounts of contaminating virus.

RESULTS

Attempts to transmit SV 1

In three experiments with Mung beans in water culture, inocula of tobacco necrosis virus A+SV 1 and tobacco necrosis virus B+SV 2 were tried with *Olpidia* 1, 2 and 3. *Olpidium* 3, with either virus mixture, produced numerous lesions on the Mung bean roots; there were a few lesions with tobacco necrosis virus A+SV 1 and *Olpidium* 2 but none with the other combinations. Sap extracted from these infected roots gave no sign of containing satellite virus particles when examined electron-microscopically, although there were tobacco necrosis virus particles roughly proportional in number to the density of the lesions on the roots. In one experiment, however, with tobacco necrosis virus B+SV 1 and *Olpidium* 3, satellite virus particles were as plentiful as tobacco necrosis virus particles, but the presence of SV 1 could not be substantiated by serology. Later work suggested that the particles were probably SV 3 and that the virus inoculum was contaminated with this strain, which is only slightly related serologically to SV 1 and would not have precipitated with the dilute antiserum to SV 1 used in our tests. Three attempts were made to transmit SV 1 by *Olpidia* 1, 2 and 3 to Mung bean, lettuce and tobacco plants in sand culture, but we could not demonstrate SV 1 in any of the plants infected with tobacco necrosis virus. Had SV 1 been transmitted it should have been detectable electron-microscopically and serologically (the sensitivity of electron microscopy in detecting virus is of the same order as that of serology) because Kassanis & Nixon (1961) found that naturally infected tobacco roots could contain 10^{11} particles of SV 1 per ml. of sap. Failure could not be attributed to using the wrong activator because tobacco necrosis viruses A and B were strains occurring with SV 1 in naturally infected plants. A possible explanation of the failure could be that the SV 1 used has been transmitted mechanically at Rothamsted for the last 25 years and may thereby have lost its ability to be transmitted by *Olpidium* zoospores. Several viruses have been reported to have lost the ability to be transmitted by an insect vector after prolonged mechanical transfer. Attempts were therefore made to obtain new isolates of SV 1 from naturally infected plants grown in unsterilized loam at Rothamsted, but in contrast to past experience (1960–2), when SV 1 was the only strain found, none of the five isolates of satellite virus tested was identical with SV 1. When the new isolates were compared with SV 1 by precipitation-in-gel tests using SV 1 antiserum, the new isolates formed a weak line intersecting a

pronounced line formed by SV 1, thus making a large spur. These results suggested a considerable serological difference between SV 1 and the new isolates, but a more interesting difference was that the new viruses were transmissible by *Olpidium* 3. Each new isolate occurred together with tobacco necrosis virus A.

The separation of SV 2 and SV 3 from the new isolates

There was no obvious serological difference between the 5 isolates of satellite virus, so only one was used for further work. The inoculum was passed several times through single lesions in tobacco plants and bulked in tobacco. The viruses were extracted, purified, and satellite virus separated from tobacco necrosis virus by two centrifugations in sucrose gradients. The new strain of satellite virus was then mixed with tobacco necrosis virus B and passed again through several single lesions. Tobacco necrosis virus B was used because it denatures during precipitation by ammonium sulphate and satellite virus is more easily separated than when the activator is tobacco necrosis virus A. An antiserum was prepared against this purified preparation of the new strain, which was called SV 2. To our surprise, after single-lesion purification and change of activator, the new strain was no longer transmissible by *Olpidium* 3, even with tobacco necrosis virus A in the inoculum, whereas from the unselected virus culture we could still transmit a satellite virus. The significance of this result was shown by precipitation-in-gel tests with antisera to SV 1 and SV 2 and sap from tobacco roots infected with the unselected virus. The sap produced a faint line with both sera and pronounced spurs were formed by SV 1 and SV 2 when placed in wells next to the sap. This result suggested that the initial culture contained not ony SV 2 but also a third strain, which we call SV 3, that was eliminated during passage through single lesions.

The precipitation titre of sap from tobacco roots infected with SV 3 varied from 1/4 to 1/32 indicating considerable multiplication of the SV 3. It seemingly multiplies much less in tobacco leaves, for sap from leaves with many necrotic local lesions (tobacco necrosis virus A activator) caused by inoculating with root sap did not contain enough SV 3 to be detected either serologically or by electron microscopy until the virus was concentrated more than 50 times. The presence of SV 3 in the inoculated tobacco leaves was also shown by the infection of tobacco roots inoculated with leaf sap and *Olpidium* 3. This result emphasizes that transmission by *Olpidium* is much more sensitive than other methods of detecting satellite or tobacco necrosis viruses. Passage of SV 3 through tobacco leaf and back to tobacco root did not diminish its ability to multiply in the root.

The serological and other properties of SV 2 and SV 3 will be described elsewhere. It is only necessary to mention here that the three strains differ considerably antigenically and are easily identified by precipitation-in-gel tests. Also, in precipitation tests in tubes, the antisera, when diluted 1/50, reacted only with their homologous antigens.

Transmission of SV 3 and SV 2

Of the *Olpidia* we have tested, only isolate 3 transmitted SV 3 and it did so even from a mixture of satellite strains. We have already mentioned that SV 3 multiplies little in tobacco leaves, yet when sap containing only traces of SV 3, from leaves inoculated with tobacco necrosis virus A + SV 2 + SV 3, was used as inoculum for roots

with *Olpidium* 3, only SV 3 was transmitted. SV 2 was not transmitted by *Olpidia* 1, 2 or 3 but the fact that it came from naturally infected plants suggested that in the unsterilized glasshouse soil there had been another *Olpidium* able to transmit it. An attempt to obtain such an *Olpidium* was made and two fungi were isolated from root washings from each of two tobacco plants naturally infected with tobacco necrosis virus and SV 2. One of the two isolates, called *Olpidium* 6, transmitted SV 2. This fungus has been propagated in a series of tobacco plants for more than a year and still carries both viruses, tobacco necrosis virus A and SV 2. The fungus was freed from viruses by inoculating plants with a zoospore suspension so diluted as to infect only an occasional plant, and several plants were infected by *Olpidium* without virus. Virus-free *Olpidium* 6 was also obtained by inoculating plants with acid-treated resting spores (Campbell, 1962). However, we have so far failed to transmit SV 2 by adding it with tobacco necrosis virus A to the virus-free *Olpidium* 6. Two explanations for this are suggested. First, in freeing the fungus from virus we may have selected a strain of *Olpidium* that does not transmit SV 2. Secondly, *Olpidium* 6 rarely yields dense suspensions of zoospores, usually giving 4×10^6 or fewer per plant compared with 4×10^7 often obtained with other strains of the fungus. As we have noted, we have had fewer transmissions in these experiments, particularly with sand cultures, than previously with tobacco necrosis virus. Why this has been so is not clear, but smaller spore concentrations may be among the factors that have caused failure of transmission by the virus-free *Olpidium*. Altogether we have made 36 experiments, in 4 of which no virus was transmitted, in 9 only tobacco necrosis virus, and in 23 tobacco necrosis virus and satellite virus.

DISCUSSION

The results show that satellite virus seems to be transmitted by *Olpidium* and in the same circumstances as is tobacco necrosis virus, namely transmission occurs when susceptible host roots are placed in a liquid medium containing infective *Olpidium* zoospores together with satellite virus and its activating tobacco necrosis virus. In about 90 % of all our experiments tobacco necrosis virus was transmitted, and in about 70 % of those satellite virus was also. If conditions were such that transmission of tobacco necrosis virus sometimes failed, it is not surprising that the conditions for transmission of two viruses were fulfilled rather less frequently. A totally different though less likely explanation would be that satellite virus is transmitted by an associated organism that fluctuates in numbers independently of *Olpidium*. The Olpidium cultures are free from other obvious root parasites but include micro-organisms that live on or around the roots. By studying the manner in which *Olpidium* zoospores seemed to transmit tobacco necrosis virus, this possibility was largely eliminated so far as tobacco necrosis virus was concerned. However, results with satellite virus have not been so helpful. If we accept that *Olpidium* transmits tobacco necrosis virus, the fact that satellite virus is found only together with tobacco necrosis virus, which, so far as is known, is necessary for satellite virus to multiply, is probably the strongest argument that *Olpidium* is the vector of both viruses.

Just as transmission of tobacco necrosis virus by *Olpidium* depends on the right combination of host species, fungus isolate and virus strain (Kassanis & Macfarlane, 1965), so the results with satellite virus suggest that this virus might have similar specific relationships. Unfortunately, experimental difficulties have prevented our

discovering more than a small part of what is probably a complicated situation. *Olpidium* 3 has always been the most infective isolate, so specific effects may be relative rather than absolute. As tobacco plants were used in most experiments, we do not know whether there is host specificity. Whether the strain of activator is important is likewise unknown. The possibility that the SV 1 culture has, after manual transfer for more than 20 years, lost ability to be transmitted by *Olpidium* cannot be checked until a new isolate of this strain is found. The SV 1 strain was prevalent in the unsterilized soil in use at Rothamsted around 1960 when many roots were examined, and we must assume that there was a fungus able to transmit it.

The greater concentrations attained by SV 3 in roots than in leaves of tobacco is noteworthy. Metabolic differences between root and leaf are considerable and there are probably further differences when the root is infected by *Olpidium*. Presumably these differences are important for the multiplication of SV 3.

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