Specificity and Site of in vitro Acquisition of Tobacco Necrosis Virus by Zoospores of Olpidium brassicae

By J. H. M. TEMMINK,* R. N. CAMPBELL and P. R. SMITH†

Department of Plant Pathology, University of California, Davis 95616

(Accepted 31 July 1970)

SUMMARY

Four single-sporangium isolates of Olpidium brassicae were inoculated on 14 different plant species to determine their ability to infect and reproduce in each host and their ability to transmit two tobacco necrosis virus isolates. Two O. brassicae isolates, one from lettuce and one from tomato, were able to reproduce in most hosts and transmitted both tobacco necrosis virus isolates to all hosts. By contrast, a mustard isolate reproduced in only six species and was not a vector of tobacco necrosis virus. An oat isolate reproduced in six species (three in common with the mustard) and was a poor vector, transmitting one tobacco necrosis virus isolate to only two hosts and not transmitting the other tobacco necrosis virus isolate to any host.

Zoospores of the different fungus isolates were mixed with suspensions of virus, washed, negatively stained, and examined in the electron microscope. Virus adsorbed tightly to the surface membranes (plasmalemma of the body and axone- mal sheath) of zoospores of isolates that transmitted it, but not to those of the non-vector (mustard) isolate. Zoospores of the poor vector (oat) isolate adsorbed fewer particles of the tobacco necrosis virus isolate they transmitted than did those of good vectors and did not adsorb the other tobacco necrosis virus isolate. Most, but not all, of the observed specificity of transmission of tobacco necrosis virus seems to be associated with the ability or inability of zoospores to adsorb the virus on their surfaces. None of these isolates adsorbed particles of turnip yellow mosaic, tomato bushy stunt or cucumber necrosis viruses. O. brassicae zoospores also adsorbed satellite virus particles. Zoospores of Olpidium cucurbitacearum adsorbed particles of cucumber necrosis virus, but not of tobacco necrosis virus, to their surfaces. Thus, in vitro acquisition consisted of a tight adsorption of virus to the zoospore surface membranes in each of the three known instances of this type of relationship.

INTRODUCTION

As information on the transmission of tobacco necrosis virus (TNV) by Olpidium brassicae (Wor.) Dang. has accumulated, several hypotheses have been considered to explain how the zoospores of the fungus transmit the virus. These have been reviewed and modified by Campbell & Fry (1966) who proposed that TNV is acquired in vitro by zoospores, is tightly bound to the zoospore surface where it can be inactivated by concentrated antiserum to TNV (Kassanis & Macfarlane, 1964), and is external to the resting spores. Although

* Present address: Laboratory for Virology, State Agricultural University, Wageningen, The Netherlands.
† Present address: Victorian Plant Research Institute, Burnley, Victoria 3121, Australia.
zoospores and virus have generally been mixed *in vitro* for transmission experiments, a similar exposure of zoospores to virus independently released from roots occurs in soil water about the roots of undisturbed plants (Smith, Campbell & Fry, 1969). Thus, ‘*in vitro acquisition*’ is used as a general term for this type of virus + fungus relationship even though it may occur in the soil.

There are few other instances of *in vitro* acquisition of plant viruses by fungi. Dias (1970) reported that cucumber necrosis virus (CNV) was transmitted by zoospores of *Olpidium cucurbitacearum* Barr & Dias that had been mixed with a suspension of the virus. There was no evidence as to the tightness of the adsorption because zoospores died during repeated centrifugations. Some strains of satellite virus (SV) were reported to be transmitted after mixing with an activating TNV and zoospores of certain *O. brassicae* isolates *in vitro* (Kassanis & Macfarlane, 1968).

Specificity in ability of different cultures of *O. brassicae* to transmit TNV has been reported. W. P. Mowat [unpublished data cited by Teakle & Hiruki (1964)], Teakle & Hiruki (1964), and Kassanis & Macfarlane (1965) showed that crucifer cultures did not transmit TNV, whereas lettuce cultures did. Host-virus or host-fungus interactions were apparently not responsible for the specificity observed by Teakle & Hiruki (1964) who concluded that the zoospores of the crucifer strain were less efficient in acquiring TNV than those of the lettuce strains. On the other hand, Kassanis & Macfarlane (1965) emphasized host-fungus interactions as the critical factor because there was a great inhibition of TNV multiplication in cress seedling roots if a crucifer culture of *O. brassicae* was inoculated within 3 hr of the inoculation of TNV by a transmitting culture of *O. brassicae* from lettuce. Mowat (1968) showed that immobile zoospores and virus have a net negative charge. Since the net negative charge of non-vector zoospores was less than that of vector zoospores, he concluded that there was no electrostatic barrier to prevent virus attachment to non-vector zoospores and that perhaps non-vector zoospores held the virus more securely and failed to release it into the host cell.

Most studies with *O. brassicae* have utilized mass cultures that are satisfactory for demonstrating the behaviour of a heterogeneous population, but are unsuitable for studies in physiological specialization, e.g. the host range of the fungus. Thus, the wide host ranges given for mass cultures by, for example, Garrett & Tomlinson (1967) may be because the cultures are mixtures of several biotypes, all of which can infect lettuce. Only Sahtiyanci (1962) has used single-sporangium isolates. Her crucifer isolate infected cabbage, spinach, egg-plant, sugar beet and *Capsella bursa-pastoris*, whereas her lettuce isolate infected lettuce, tobacco, egg-plant, sugar beet, *C. bursa-pastoris* and occasionally tomatoes, spinach, cabbage and Gramineae.

This paper reports studies on the host ranges of four single-sporangium isolates of *O. brassicae* and their ability to transmit six TNV isolates. In addition, viruliferous zoospores were examined in the electron microscope to find the site of *in vitro* acquisition. Specificity of transmission was shown to result in part from whether the virus was or was not adsorbed to the zoospores. Some of the results have been published in abstract (Temmink & Campbell, 1969b). *In vitro* acquisition of other viruses by their fungus vectors was briefly investigated.

**METHODS**

Single-sporangium isolates of *Olpidium brassicae* were prepared by the technique of Lin *et al.* (1970) and one isolate from each of four mass cultures was selected for the transmission and host-range experiments. These single-sporangium isolates will be named according to the host from which they were originally isolated. The lettuce and tomato isolates were derived
Virus acquisition by fungus zoospores

from those used by Smith et al. (1969) and were maintained in lettuce (Lactuca sativa L. ‘Climax’). The mustard isolate was obtained from Brassica nigra (Campbell, 1965) and maintained in mustard (Brassica campestris L. Perviridis group ‘Tendergreen’). The oat isolate was trapped in roots of oats (Avena sativa L. ‘Sierra’) grown in soil collected near the source of the mustard isolate and was maintained in oat. The techniques for maintaining the isolates and producing zoospore suspensions have been described (Smith et al. 1969). Zoospores were suspended in either 0.05 M-glycine-NaOH at pH 7.6 or in tap water, and such suspensions contained 0.5 to 1.5 x 10⁶ zoospores/ml. An isolate of O. cucurbitaecearum furnished by H. F. Dias was maintained in the roots of cucumber (Cucumis sativus L. ‘National Pickling’).

The two most frequently used tobacco necrosis virus isolates, New Zealand (NZ-TNV) and tomato (1-TNV), were described by Smith et al. (1969). Partially purified preparations were made as described by Campbell & Fry (1966), except that the heating step was not used. Other isolates used occasionally were Kassan’s A (KA), Kassan’s D (KD), AC-36 and AC-42 which were obtained from J. K. Uyemoto and purified in the same manner as other isolates. Purified satellite virus c (SV-c), isolated from a mixture with AC-36 (Uyemoto, Grogan & Wakeman, 1968), was obtained from J. K. Uyemoto. Cucumber necrosis virus (CVN) was supplied by H. F. Dias and partially purified in the same manner as TNV. The viruses were added to zoospore suspensions in vitro to give a final virus concentration of approximately 2 µg./ml.

The host range and ability to transmit TNV were determined for each isolate of O. brassicae in experiments using two replicates of 14 species of host plants and four treatments, i.e. O. brassicae alone, TNV alone, O. brassicae + TNV, and nil. The host plants, grown from seed in pasteurized quartz sand in 30- or 100-ml. plastic beakers, were inoculated by pipetting 5 ml. of test solution on to the sand 4 to 10 days after the seeds were sown (Campbell & Fry, 1966). The number of seedlings was approximately equal for each host species but varied between ten for large-seeded hosts such as cowpea to about fifty for small-seeded hosts such as lettuce. The plants were incubated at 16°C for 4 days. At this time, plants in each replicate were washed free of sand and placed in 3 ml. of tap water (6 ml. for seedlings such as cowpea with large roots). The susceptibility of each host to O. brassicae was determined from the plants inoculated with O. brassicae alone. Five to 10 min. after the roots had been placed in tap water, this fluid was examined for motile zoospores. If no zoospores were detected, three roots were removed and microscopically examined for sporangia that might not have matured and released zoospores. If the number of zoospores released or the number of sporangia found microscopically was estimated as equal to those from the maintenance host of the isolate, the test plant was considered a satisfactory host. If few zoospores were released and few mature sporangia were present, the test plant was considered a poor host. If there were no zoospores or sporangia, the test plant was rated a non-host. TNV transmission was assayed by triturating the test plant roots in tap water and mechanically assaying the sap on four primary leaves of bean (Phaseolus vulgaris L. ‘Bountiful’). The results are expressed as the number of local lesions/leaf averaged for both replicates; lesions in excess of 150/leaf were not counted and were recorded as 150.

The test host range included, in addition to the species used to maintain the Olpidium isolates, Parris Island Cos lettuce, cress (Lepidium sativum L. ‘Curlicress’), wheat (Triticum aestivum L. ‘Michigan Amber’), tomato (Lycopersicon esculentum Mill. ‘Improved Pearson’), cowpea (Vigna sinensis (Stickm.) Savi ex Hassk. ‘Blackeye’) pea (Pisum sativum L. ‘Alaska’), cucumber (Cucumis sativus L. ‘National Pickling’), spinach (Spinacia oleracea L. ‘Viroflay’),
To study adsorption of viruses to zoospores, a zoospore suspension was prepared in tap water (25 to 50 ml). Partially purified virus was added to a final concentration of about 2 μg/ml and the mixture was kept 5 to 10 min. at room temperature (about 22°). The zoospores were washed three times as described (Campbell & Fry, 1966) except that O. cucurbitacearum was centrifuged each time at only 5000 rev./min. for 5 min. After the final sedimentation, the zoospores were resuspended in about 2 ml. of tap water and a drop of the suspension was put on to a 300-mesh copper grid covered with a carbon-coated collodion film. The grid was inverted over an open vial containing 4% osmic acid for 1 to 2 min. to kill and fix the zoospores. A similar sized drop of 4% uranyl acetate was added and the excess mixture was removed with filter paper. The grid was air-dried and examined in an RCA EMU-3H electron microscope.

RESULTS

Host range of Olpidium brassicae isolates and specificity of TNV transmission

The host range and ability to transmit two isolates of tobacco necrosis virus were tested with four isolates of Olpidium brassicae (Table 1). The host range of each fungus isolate was determined from the plants inoculated with Olpidium alone in at least two separate experiments. In assays for TNV transmission none of the seedlings inoculated with Olpidium alone or the uninoculated controls yielded any TNV lesions, and for brevity these results have been omitted from Table 1. The seedlings inoculated with TNV alone usually yielded no lesions, but an occasional lesion was found on some leaves of the assay plants and this ‘background virus’ was subtracted from the number of lesions from seedlings inoculated with Olpidium and TNV.

The lettuce and tomato isolates infected all 14 host species, although they reproduced poorly in a few (Table 1). Both of these fungus isolates transmitted both TNV isolates to all hosts including some in which they multiplied poorly. A high titre of virus was recovered from most hosts. Although little virus was recovered from mustard or sugar beet, they were regarded as having been infected with TNV by zoospores because somewhat more than background virus was recovered in the assay, and we suspected that their saps might be inhibiting the infectivity of the virus they contained. Experiments were therefore done to test for inhibitors in the saps prepared from healthy roots in the same manner as done in the transmission trials. In one experiment constant amounts of TNV were mixed with lettuce root sap, sugar beet root sap, or buffer, and the mixtures assayed on bean leaves. The average numbers of local lesions/leaf were: buffer, 68; lettuce root sap, 92; sugar beet root sap, 1. In another trial constant amounts of TNV were added to buffer or dilutions of sap from healthy mustard roots and four half-leaf comparisons were done between each sap dilution and the buffer control. The reduction in the number of TNV lesions was 84% for undiluted sap, 74% with the 1:2 dilution, 32% with the 1:4 dilution, and 4% with the 1:8 dilution. The small infectivity of saps from sugar beet and mustard, therefore, is due in large part to inhibitors that interfere with the mechanical transmission of the virus to the assay hosts.

The mustard isolate reproduced well in five hosts, poorly in one, and apparently did not infect the remainder (Table 1). The assays for TNV transmission generally yielded no TNV lesions except in a few cases (Table 1). This amount of TNV is of the same magnitude as the background TNV and is regarded as insignificant. Thus, the present mustard isolate does not seem to be a vector of TNV and is comparable to the crucifer isolates used by others (Kassanis & Macfarlane, 1965; Teakle & Hiruki, 1964).
Table 1. Transmission of two tobacco necrosis virus isolates by four single sporangium isolates of Olpidium brassicae

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce, Climax</td>
<td>+</td>
<td>122</td>
<td>110</td>
<td>+</td>
<td>150</td>
<td>98</td>
<td>–</td>
<td>0.5</td>
<td>0.4</td>
<td>+</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lettuce, Parris Island Cos</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>–</td>
<td>0.7</td>
<td>0</td>
<td>–</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>Mustard</td>
<td>P</td>
<td>2</td>
<td>10</td>
<td>+</td>
<td>22</td>
<td>13</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Oats</td>
<td>+</td>
<td>41</td>
<td>66</td>
<td>+</td>
<td>29</td>
<td>27</td>
<td>–</td>
<td>0.5</td>
<td>0.2</td>
<td>+</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Wheat</td>
<td>+</td>
<td>139</td>
<td>132</td>
<td>+</td>
<td>64</td>
<td>150</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tomato</td>
<td>P</td>
<td>108</td>
<td>149</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>–</td>
<td>0.1</td>
<td>0</td>
<td>–</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cowpea</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>+</td>
<td>150</td>
<td>124</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>101</td>
<td>4</td>
</tr>
<tr>
<td>Pea</td>
<td>+</td>
<td>16</td>
<td>43</td>
<td>+</td>
<td>59</td>
<td>13</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cucumber</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>P</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>45</td>
<td>0.1</td>
</tr>
<tr>
<td>Spinach</td>
<td>+</td>
<td>29</td>
<td>94</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Sugar beet</td>
<td>+</td>
<td>3</td>
<td>2</td>
<td>+</td>
<td>3</td>
<td>6</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Celery</td>
<td>P</td>
<td>53</td>
<td>82</td>
<td>+</td>
<td>93</td>
<td>131</td>
<td>–</td>
<td>1.5</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. glutinosa</td>
<td>+</td>
<td>124</td>
<td>150</td>
<td>P</td>
<td>150</td>
<td>150</td>
<td>–</td>
<td>0</td>
<td>0.1</td>
<td>–</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Tobacco</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>+</td>
<td>150</td>
<td>150</td>
<td>+</td>
<td>0.4</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Olpidium infection and reproduction in the respective hosts inoculated with Olpidium free of TNV. + = Normal reproduction, zoospores discharged in quantity; P = infected but reproduced poorly, few sporangia produced, zoospores not usually detected; – = no evidence of infection/or reproduction.
† Transmission of two isolates of TNV indicated by average number of local lesions/bean leaf/replicate produced when four 'Bountiful' bean leaves were inoculated with homogenized roots of bait plants from Olpidium+TNV treatments in 3 or 6 ml of tap water. The average number of local lesions produced by assay plants when inoculated with 'TNV without Olpidium' treatments of the same host were subtracted; there were no local lesions in assays of bait plants from 'Olpidium only' and 'uninoculated' controls.
The oat isolate multiplied in six hosts, three of which were also hosts of the mustard isolate (Table 1). Because the amount of TNV obtained from the bait plants was less than that obtained with the transmitting and more than with the non-transmitting isolates of the fungus, each experiment with each of the two TNV isolates was done twice. Similar results were obtained in each experiment, and the results in Table 1 represent the average. The oat isolate transmitted NZ-TNV to cowpea, cucumbers and possibly to Parris Island Cos lettuce. The few lesions obtained from other hosts and in the T-TNV transmission experiments are similar to those obtained with the mustard isolate and are regarded as insignificant. The oat isolate is distinct from the other isolates in its host range and is a poor vector of TNV.

Each isolate was further tested for its vector capability in one experiment using four additional TNV isolates and six plant species. Because in other experiments cress was found to lack an inhibitor affecting the number of lesions on bean leaves, it was substituted for Tendergreen mustard. As in previous experiments, the controls were negative except for background TNV in the TNV alone treatment, and the results from the Olpidium + TNV treatments are shown in Table 2. The lettuce and tomato isolates transmitted all four TNV isolates to at least some hosts, but few lesions were obtained in some assays. The mustard isolate did not transmit any TNV isolates to any host and the oat isolate was a poor vector, transmitting only Kassanis' A strain to cowpea.

An experiment was done to test the possibility that the host in which zoospores were produced might affect their ability to transmit TNV. Because the tomato isolate multiplied satisfactorily in Tendergreen mustard, which is the maintenance host for the non-vector mustard Olpidium, it was inoculated to this host and serially transferred at irregular intervals for 3 months. At the end of this time, the tomato isolate maintained in mustard was compared with the same isolate from lettuce and with the mustard isolate for transmission of NZ-TNV to lettuce, mustard or tomato. The tomato isolate whether maintained in mustard or lettuce, infected all three hosts and transmitted TNV to lettuce and tomato (Table 3). A less concentrated TNV suspension was used in this experiment, so there were fewer TNV lesions than usual when the bait plants were tested. The mustard isolate infected only mustard and did not transmit TNV to any host. Thus, reproduction of the tomato isolate in mustard did not affect its ability to transmit TNV or to reproduce in lettuce or tomato.

Site and specificity of acquisition of TNV by Olpidium zoospores

When zoospores of the lettuce or tomato isolates were mixed with either NZ-TNV or T-TNV and then washed three times, many virus particles remained adsorbed to the outer membrane, i.e. the plasmalemma of the zoospore body (Fig. 1) and the axonemal sheath (Fig. 2) which are known to be continuous (Temmink & Campbell, 1969a). There were a few virus particles that were not attached to the zoospores but were close to the zoospore, suggesting that they had become detached during fixation, staining and drying; this type of particle will be referred to as detached virus. If zoospores were not washed, there were many particles on the zoospore as well as scattered over the grid with no spatial relationship to the zoospore; the latter will be referred to as excess virus (Fig. 3).

Washed zoospores of the mustard isolate that did not transmit TNV had no adsorbed, detached, or excess virus (Fig. 4, 5). Zoospores of the oat isolate exposed to NZ-TNV and washed had a small but significant number of virus particles attached to the zoospore plasmalemma (Fig. 6) and the axonemal sheath (Fig. 7). There were detached virus particles also. When zoospores of the oat isolate were mixed with T-TNV no adsorbed virus particles could be found on the zoospores.
Table 2. Transmission of additional tobacco necrosis virus isolates by four Olpidium brassicae isolates

<table>
<thead>
<tr>
<th>Bait plants</th>
<th>Lettuce</th>
<th>Tomato</th>
<th>Mustard</th>
<th>Oat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KA</td>
<td>KD</td>
<td>AC-36</td>
<td>AC-42</td>
</tr>
<tr>
<td>Lettuce</td>
<td>9†</td>
<td>61</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>Cress</td>
<td>62</td>
<td>77</td>
<td>27</td>
<td>122</td>
</tr>
<tr>
<td>Oat</td>
<td>18</td>
<td>81</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Tomato</td>
<td>25</td>
<td>54</td>
<td>5</td>
<td>137</td>
</tr>
<tr>
<td>Cowpea</td>
<td>73</td>
<td>150</td>
<td>86</td>
<td>126</td>
</tr>
<tr>
<td>Cucumber</td>
<td>38</td>
<td>26</td>
<td>0</td>
<td>123</td>
</tr>
</tbody>
</table>

* KA = Kassanis' strain A; KD = Kassanis' strain D; AC-36, AC-42 = American Type Culture Collection isolates 36 and 42
† Infectivity of sap from bait plant roots. For details see Table 1, footnote †

Table 3. Effect of fungus increase host on transmission of TNV by two isolates of O. brassicae

<table>
<thead>
<tr>
<th>Fungus isolate</th>
<th>Increase host</th>
<th>TNV transmission to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lettuce</td>
</tr>
<tr>
<td>Tomato</td>
<td>Lettuce</td>
<td>56*</td>
</tr>
<tr>
<td>Tomato</td>
<td>Mustard</td>
<td>38</td>
</tr>
<tr>
<td>Mustard</td>
<td>Mustard</td>
<td>0</td>
</tr>
</tbody>
</table>

* Infectivity of sap from bait plant roots. For details see Table 1, footnote †
Fig. 1, 2, 3. Zoospores of lettuce isolate of *O. brassicae* and NZ strain of tobacco necrosis virus.

Fig. 1, 2. Zoospores washed free of excess virus before fixing and staining showing virus adsorbed to plasmalemma of body (Fig. 1) and to axonemal sheath (Fig. 2). Fig. 3. Unwashed zoospores showing excess virus not adsorbed to zoospore.
Fig. 4, 5. Washed zoospores of mustard isolate of O. brassicae showing absence of adsorbed tobacco necrosis virus.

Fig. 6, 7. Washed zoospores of oat isolate of O. brassicae showing the few adsorbed particles of NZ strain of tobacco necrosis virus.
As controls, experiments were done in which zoospores of the lettuce, tomato, and mustard isolates were mixed with partially purified preparations of three polyhedral plant viruses: turnip yellow mosaic virus, tomato bushy stunt virus, and cucumber necrosis virus. After washing no particles of these viruses could be found.

**Site of acquisition of other fungus-transmitted viruses acquired in vitro**

Since adsorbed TNV could be seen on *O. brassicae* zoospores, other viruses acquired *in vitro* and transmitted by fungus vectors were investigated. Zoospores of *O. cucurbitacearum* were mixed with cucumber necrosis virus (CNV) and washed. There were virus particles adsorbed to the plasmalemma and axonemal sheath as well as detached and lying near the fungus (Fig. 8, 9). Particles of CNV seemed to become detached more easily from this vector than do those of TNV from *O. brassicae*. When zoospores of *O. cucurbitacearum* were mixed with TNV and washed, no virus was adsorbed by the zoospores.

Zoospores of lettuce *Olpidium* were tested with satellite virus C (SV-c) and strains of TNV. SV-c was adsorbed on zoospores when it was the only virus added (Fig. 10). SV-c and AC-36 strain of TNV were both adsorbed when zoospores were exposed to a mixture of both viruses (Fig. 11). Similar results were obtained when zoospores were exposed to a mixture of SV-c and NZ-TNV. To test whether one virus might block the adsorption site of the other, batches of zoospores were exposed to either NZ-TNV or SV-c for 10 min before the other virus was added. Ten min later the zoospores were washed and prepared for examination. Both TNV and SV were adsorbed to zoospores regardless of the sequence in which the viruses were mixed with zoospores.

**DISCUSSION**

The host-range results show the extent of physiological specialization of these four *Olpidium brassicae* isolates. Although of mycological interest, they will not be discussed further. The results also provide a partial explanation for the mode and specificity of TNV transmission. Apparently the lettuce and tomato isolates are similar to the vector isolates used by other authors, and the mustard isolate is similar to their non-vector isolates from crucifers (Kassanis & Macfarlane, 1965; Mowat, 1968; Teakle & Hiruki, 1964), whereas the oat isolate is different and is intermediate in its ability to transmit TNV.

Transmission of TNV (Tables 1, 2) is thought to require several distinct processes—acquisition of virus by zoospores, virus movement into the encysting zoospore, fungus infection, virus release into the host, and multiplication of the virus in the host. Furthermore, inhibitors that reduce the amount of virus detected in assays of host roots can obscure the results of experiments. The primary objective of this study was to determine the way viruses are acquired *in vitro* and to see whether there is any specificity in this process. The later stages of transmission will be discussed by Temmink (in preparation).

The electron micrographs of vector isolates of *O. brassicae* confirm the hypothesis of *in vitro* acquisition proposed by Campbell & Fry (1966). When vector zoospores acquire TNV, it is tightly adsorbed on to their outer membrane—the plasmalemma of the body and the axonemal sheath. The observations of mixtures of SV and *O. brassicae* and of cucumber necrosis virus and *O. cucurbitacearum* show that these viruses also adsorb to the outer membrane of the zoospore. The mechanism by which the virus is adsorbed is not known. If motile zoospores have the same electrostatic charges found on immobile and presumably dead zoospores (Mowat, 1968), there should be more virus adsorbed to zoospores of the mustard isolate than to those of vector isolates. This has not been observed, suggesting that
Fig. 8, 9. Washed zoospores of *O. cucurbitacearum* showing particles of cucumber necrosis virus which presumably became detached when samples were prepared for electron microscopy.

Fig. 10. Washed zoospore of lettuce isolate of *O. brassicae* showing adsorbed satellite virus particles.

Fig. 11. Washed zoospore of lettuce isolate of *O. brassicae* showing adsorbed particles of satellite and AC-36 tobacco necrosis viruses.
electrostatic charges are not directly responsible, but that other forces are involved in the adsorption of virus to zoospores, as also considered by Mowat (1968).

The failure of zoospores of the mustard isolate to transmit TNV is correlated with their inability to adsorb virus particles in vitro. The suggestion that vector specificity is determined by responses of host cells to different Olpidium isolates (Kassanis & Macfarlane, 1965) may have application in special circumstances, but it does not explain the failure of the mustard isolate to acquire TNV. Likewise, the suggestion that non-vector zoospores acquire TNV, though less efficiently than vector zoospores (Teakle & Hiruki, 1964), was not confirmed. We have done experiments of the type used by Teakle & Hiruki (1964) involving bursting of washed zoospores to release ‘bound’ virus that is subsequently acquired and transmitted by fresh zoospores. Although we have obtained similar results, we believe this type of experiment does not discriminate between virus released from zoospores, virus adsorbed or trapped by debris, and small amounts of excess virus. We therefore base our conclusion on the direct examination of zoospores.

The oat isolate is intermediate between the lettuce (or tomato) and the mustard isolates in its efficiency as a vector of TNV. The causes of specificity may be more complex in this case. Zoospores of the oat isolate seem to have fewer and perhaps more specific adsorption sites, and therefore seem to adsorb fewer particles of some virus isolates, e.g. NZ-TNV, than do zoospores of the lettuce isolate. Therefore, their failure to transmit NZ-TNV to all hosts is ascribed to their acquisition of less virus and to failure in later steps of virus transmission. The low concentration of detached particles observed near zoospores of the oat isolate indicates that they do not adsorb large quantities of virus particles that later become detached during processing for electron microscopy. Failure to transmit other TNV isolates to any host is ascribed to failure to acquire any virus particles.

Other viruses transmitted by fungus zoospores are apparently also acquired in vitro by surface adsorption. With SV and O. brassicae the adsorption seems as tight as with TNV and O. brassicae. With CNV and O. cucurbitacearum there are many detached particles, indicating a weaker attachment than that observed for O. brassicae, or a more disruptive change in the membrane structure during processing for electron microscopy. Nevertheless, our results confirm Dias’ (1970) hypothesis that the CNV-O. cucurbitacearum relationship is more similar to that in TNV-O. brassicae than to that in viruses such as lettuce big vein or tobacco stunt which seem to be acquired in vivo and borne internally by O. brassicae.

Staining revealed virus particles on the zoospores in two slightly different ways. When the zoospore body and axoneme were in a thick layer of stain, only the virus particles lying along the sides, where most of the stain was removed, were seen (Fig. 8, 9), perhaps the stain penetrated into the zoospore body or axoneme in these cases. When there was a thin covering of stain on the zoospores, the virus particles were negatively stained on the surface of the zoospore (Fig. 1, 2, 6, 7). In other instances, no adsorbed virus could be seen because of unfavourable staining: either there was too little stain or too much and the zoospores were totally submerged in an electron opaque layer. These poor staining conditions rarely affected all the areas of a grid, and with practice some control over the amount of stain left on a grid was possible. Nevertheless, the results are based on observations on more than 100 zoospores on each of 2 to 5 grids made in different trials.

Support by Grant AI-07864 from the U.S. Public Health Service is gratefully acknowledged.
Virus acquisition by fungus zoospores

REFERENCES


(Received 23 June 1970)