Purification, Serology and Nucleic Acid of Oat Sterile Dwarf Virus Subviral Particles

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SUMMARY

Oat sterile dwarf virus (OSDV) subviral particles (SVPs) were purified from roots and stem bases of Lolium multiflorum. Electron microscopy of the preparations showed B-spiked SVPs typical of Fijiviruses (Fiji disease and maize rough dwarf-like viruses). Two sera with titres in gel-diffusion tests of 1/512 and 1/2048 were prepared against the SVPs. The sera did not react with the SVPs of maize rough dwarf and pangola stunt viruses when tested by gel-diffusion and immunoelectron microscopy. The first serum did not react with virus dsRNA or poly (1).poly(C) and the second serum had a titre of 1/8 with each of these. Polyacrylamide gel electrophoresis of the nucleic acid from purified OSDV SVPs revealed ten dsRNA segments though only nine of these were resolved as separate bands in dsRNA extracted directly from infected plants. The genome pattern was broadly similar to but distinct from those of the known Fijiviruses. The RNA of the serologically related Arrhenatherum blue dwarf virus (ABDV), extracted directly from plants, appeared identical to that extracted directly from OSDV-infected plants. Both OSDV and ABDV were transmitted by the planthopper vector Javesella pellucida despite, in the case of OSDV, having been maintained in vegetatively propagated plants for 5 years.

INTRODUCTION

The Fijiviruses are a group of plant-insect viruses within the Reoviridae, recently recognized as distinct from wound-tumour and rice dwarf viruses (Matthews, 1979). They have double-shelled virus particles 65 to 70 nm in diameter containing 10 dsRNA segments, are transmitted propagatively by planthoppers (Delphacidae) and are largely or completely confined to the vascular tissues of their plant hosts, Gramineae, where they provoke characteristic enations (see Milne & Lovisolo, 1977).

Oat sterile dwarf virus (OSDV) , widespread in Europe, is a candidate for this group as it has the typical viral and subviral particles (SVPs), is transmitted propagatively by the planthopper Javesella pellucida and causes symptoms in Gramineae which include dwarfing, dark blue-green leaf colour, extra tillering, flower suppression and small enations on the leaf veins (Catherall, 1970; Milne et al. 1975; and see Milne & Lovisolo, 1977). Two other European viruses provisionally named Lolium enation virus (LEV) and Arrhenatherum blue dwarf virus (ABDV) have similar properties. The three viruses are serologically unrelated to other known Fijiviruses (Milne & Luisoni, 1977) but nothing further was known of their interrelationships or their place within the Fijivirus group as none had been purified, no antisera existed and the virus genomes had not been studied.

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We report here on the purification of the SVPs of OSDV, the raising and testing of antiserum against these, and an electrophoretic analysis of the RNAs of OSDV and ABDV. The purification of intact virus particles was not feasible because of their instability and low concentration. The first OSDV antiserum obtained was used to show that OSDV, ABDV and LEV are serologically closely related (Milne & Lesemann, 1978).

METHODS

Viruses and RNAs. Italian ryegrass (*Lolium multiflorum*) plants infected with OSDV, obtained from Dr R. T. Plumb, Rothamsted Experimental Station, Harpenden, England, were propagated vegetatively in the glasshouse. These plants also contained a small isometric seed-borne virus, not yet characterized and present in many ryegrass seed lots; it is referred to as ryegrass spherical virus (RSV; Plumb & Misari, 1974). Tall oatgrass (*Arrhenatherum elatius*) plants naturally infected with ABDV were obtained from either Steinach, West Germany (from Dr W. Huth, Braunschweig) or Leipzig, East Germany (from Dr G. Kempiak and Professor H. Muhle, Leipzig) and vegetatively propagated or processed on receipt. *Lolium* and *Arrhenatherum* plants free from OSDV and ABDV were grown from seed.

Particles of maize rough dwarf virus (MRDV) and pangola stunt virus (PSV), (Milne, 1977; Boccardo *et al.* 1979) were used in comparative serological tests. RNA was prepared from MRDV particles according to the double-phase phenol-SDS method (see Boccardo & Milne, 1975), and poly(I).poly(C) was obtained from P-L Biochemicals Inc., Milwaukee, U.S.A.

Purification of OSDV SVPs. The progress of purification was followed by electron microscopy, and by serology when antisera became available, but not by infectivity, as the SVPs, at least of MRDV, have little or no infectivity (Milne *et al.* 1973).

All work was done at about 4 °C. About 100 g of cleaned roots and stem bases, excluding green parts, were blended with 4 vol (w/v) of extraction solution (0·4 M-phosphate buffer, pH 7·0 containing 5 mM-NaEDTA and 10 mM-Na$_2$SO$_4$) and squeezed through a nylon stocking. The filtrate was shaken with an equal vol. of Freon 113 for 30 min, then centrifuged at low speed to separate the phases. The water phase was then centrifuged at 30000 rev/min for 90 min in a Beckman rotor 30 and the pellets resuspended in extraction solution (1/30 starting vol.) The suspension was again shaken with Freon for 10 min and centrifuged at low speed, and the water phase was then centrifuged at 40000 rev/min for 45 min in a Beckman rotor 40. The pellets were resuspended three times in extraction solution and centrifuged at 12000 rev/min for 15 min, the clear supernatants (about 1·5 ml in all) being pooled. This pool was centrifuged on a pre-formed Cs$_2$SO$_4$ density gradient (linear, 20 to 60 %) in 0·1 M-phosphate buffer containing 5 mM-Na-EDTA, using a Beckman SW39L rotor at 36000 rev/min for 1 to 2 h. Two band consisting of SVPs were formed; the upper was seen in the electron microscope to contain damaged particles, the lower intact SVPs. RSV particles banded considerably higher in the tube. The lower SVP band was collected and the Cs$_2$SO$_4$ removed by dialysis or by dilution and ultracentrifugation.

Extraction of dsRNA from purified SVPs. The RNA was isolated from the SVPs by the double-phase phenol-SDS method (Boccardo & Milne, 1975).

Isolation of dsRNA directly from infected plants. As we failed to obtain enough purified particles of ABDV for RNA extraction, the following method was developed to obtain the RNA directly from infected plants. All enzymes came from Sigma Chemical Co., St Louis, Mo., U.S.A. All work was done at about 4 °C. OSDV and ABDV-free plants were processed in parallel with infected plants.

Cleaned roots and stem bases were homogenized with 3 vol. (w/v) of STE buffer (0·1 m-NaCl, 0·05 m-tris, 0·005 m-Na-EDTA, pH 7·5). SDS, preincubated protease (type VI) from
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*Streptomyces griseus*) and diethylpyrocarbonate were then added to give final concentrations of 1%, 0.1%, and 0.5% respectively and the mixture was kept at 37°C for 1 h. Bentonite (1%) was then added and the mixture shaken with 1 vol. of 90% phenol for 1 h. The phases were separated by centrifugation and the aqueous phase recovered. The organic phase was re-extracted with 1/3 the original vol. of STE buffer and the pooled aqueous phases were washed several times with chloroform–isoamyl alcohol (5:1) until no precipitates appeared at the interphase. The nucleic acids in the water phase were then precipitated with 2.5 vol. of redistilled ethanol, pelleted by low speed centrifugation, resuspended in 10 to 20 ml of 0.01 M Na-acetate, mixed with an equal vol. of 4 m-LiCl (Diaz-Ruiz & Kaper, 1977) and frozen overnight. After thawing and low speed centrifugation, the supernatant, containing mostly dsRNA, was precipitated with ethanol, resuspended in 5 ml protease reagent (0.1% protease, 0.5% SDS, 0.1 M Na-acetate) and dialysed extensively against the same solution without added protease.

The bentonite-phenol and ethanol precipitation steps were repeated and the resulting pellet resuspended in 0.01 M-tris, 0.01 M-NaCl, pH 7.1. This solution was digested for 3 h at 4°C with 1 mg/ml RNase-free DNase in 10 mM-MgCl₂ (Unger & Reichmann, 1973). The bentonite-phenol and ethanol steps were then again repeated and the pellet, resuspended in 0.01 M-tris, 0.01 M-NaCl, pH 7.1, was digested for 30 min at 37°C with 1 mg/ml amylase (α+β) in 10 mM-CaCl₂. Protease (1 mg/ml) was then added directly and digestion continued for a further 30 min at 37°C. The bentonite-phenol and ether steps were once again repeated and the pellet was washed with ethanol, then ether and dried.

**Polyacrylamide gel electrophoresis of virus RNAs.** RNA from purified SVPs or that extracted directly from infected plants was electrophoresed at room temperature in 5% polyacrylamide gels 12 cm long and 6 mm in diam., using 1.5 to 2.0 mA/tube. The gels were run with 0.04 M-tris Na-acetate-EDTA buffer, pH 7.2, for 24 h, followed by 30 h in 0.045 M-tris-Na-phosphate-EDTA buffer, pH 7.9 (Reddy et al. 1975a). They were stained in 0.01% toluidine blue O in 5% acetic acid, destained in distilled water and scanned in a Joyce-Loebl Chromoscan. Mol. wt. of the OSDV and ABDV RNA segments were estimated by comparing their mobilities with those of co-electrophoresed MRDV RNA and using the mol. wt. data for MRDV of Reddy et al. (1975a).

**Serology.** OSDV SVPs purified as described were emulsified with Freund’s adjuvant and injected into two rabbits. The first rabbit received one intramuscular and one footpad injection and was bled after 18 days; it provided the serum (serum I) used by Milne & Lesemann (1978). The second rabbit received two intramuscular injections separated by 5 days, and was bled after 1 and 2 months. The results refer to the serum obtained after 1 month (serum II) as this had the highest titre. Sera and antigens were tested by agar gel double diffusion using 0.7% Noble agar in phosphate-buffered saline, or by immunoelectron microscopy as described by Milne & Lesemann (1978).

**Electron microscopy.** Preparations were touched to grids supporting Formvar-backed carbon films. These were then rinsed with water, negatively stained with 2% aqueous uranyl acetate and viewed in a Philips EM 300 electron microscope at 60 kV.

**Vector transmission.** We tested transmission of OSDV and the ABDV from Leipzig by the planthoppers *Javesella pellucida* (the normal vector) and *Laodelphax striatellus* (an efficient vector of MRDV but not reported as a vector of OSDV or ABDV). Virus-free young nymphs were fed individually on infected plants for 3 to 5 days and, with OSDV, others were in addition injected abdominally under CO₂ anaesthesia with crude preparations of enations from infected plants. After 2 week incubation, insects were tested for infectivity by feeding them on healthy plants which were then observed for symptoms.
RESULTS

Purification

Fig. 1 shows an OSDV preparation after density gradient centrifugation; the lowest band (arrow) contained the material shown in Fig. 2. In the best preparations of OSDV SVPs, no host materials or RSV were seen by electron microscopy. The antisera obtained did not react, or reacted only up to 1/2 with healthy plant material and with purified RSV, suggesting that the SVP preparations used as immunogens contained little of these contaminants.

The SVPs could be purified from the non-green parts of plants but not from leaves because leaves yielded less OSDV and more RSV. OSDV derived from non-green material was also much less contaminated with membranes, permitting a mild clarification treatment. In phosphate buffer of low molarity, many SVPs were lost in the low speed pellets. This loss was reduced using higher molarity, but very high molarities (0.5 M upwards) damaged the SVPs. Use of Triton X-100, Nonidet P40, polyethylene glycol, urea or 2-mercaptoethanol did not seem to improve the purification. Cs₂SO₄ gave better results than other density gradient materials such as CsCl, sucrose and metrizamide.

Serology

Sera I and II reacted, in gel-diffusion tests with OSDV SVPs, to 1/512 and 1/2048, respectively. They did not react with MRDV SVPs. Serum I did not react with OSDV RNA, MRDV RNA or poly(I).poly(C), and serum II reacted with all these to 1/8. Reactions with healthy material and RSV were slight or absent (see previous section on Purification).

Immunoelectron microscopic results with serum I have been described (Milne & Lese-mann, 1978). The homologous decoration titre (the highest dilution giving antibody haloes visible in negative stain) was between 1/200 and 1/400 and the clumping titre was 1/1600. Serum II gave, with homologous SVPs, decoration up to 1/2048 and clumping up to 1/6400.
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Fig. 2. Purified OSDV B-spiked subviral particles, negatively stained in uranyl acetate.

Table 1. *Mol. wt. of the dsRNA segments of OSDV, estimated by comparison with those of MRDV co-electrophoresed in polyacrylamide gels*

<table>
<thead>
<tr>
<th>Segment</th>
<th>OSDV</th>
<th>MRDV*</th>
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<tr>
<td>1</td>
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<tr>
<td></td>
<td>18.39</td>
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</tr>
</tbody>
</table>

*Data of Reddy et al. (1975a).

The inner capsid of the SVP was decorated and probably also the B spike, though this was difficult to determine due to the presence of the layer of antibody particles attached to the inner capsid. There was no reaction with the SVPs of MRDV or PSV.

**OSDV and ABDV RNAs**

Fig. 3 shows the RNAs from purified OSDV SVPs separated by polyacrylamide gel electrophoresis and Table 1 lists the mol. wt. of these bands estimated by comparison with
those of MRDV. Visual inspection and densitometer tracings showed that 10 bands were present, each in approximately equimolar amounts. The genome segments conformed to the general Fijivirus pattern but were quite different in detail from those of other known Fijiviruses (Redolfi & Boccardo, 1974; Ikegami & Francki, 1975; Reddy et al. 1975a, b, 1976).

Double-stranded RNA extracted directly from OSDV-infected plants gave bands identical to those of RNA from purified SVPs, except that bands 7 and 8 of Fig. 3 were not resolved and ran as one band of increased intensity. The RNA from ABDV-infected plants from both East and West German sources gave faint bands indistinguishable from those of OSDV similarly extracted. No bands were found in extracts from healthy plants.

Vector transmission

The OSDV was transmitted by 41 of 97 J. pellucida (29/61 fed insects and 12/36 of those injected). None of 74 L. striatellus, fed on OSDV-infected plants, transmitted the disease.
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The Leipzig ABDV was transmitted by 19 of 43 *J. pellucida* fed on infected plants, but none of similarly fed *L. striatellus*.

**DISCUSSION**

OSDV SVPs were purified by the method described, though we were unable to purify complete virus particles. The SVPs were used to produce two essentially similar antisera against the inner capsid and (probably) the B spikes. With the first serum we have shown that OSDV, ABDV and LEV SVPs are serologically closely related (Milne & Lesemann, 1978).

With both sera we have found that the OSDV inner capsid (and probably the B spike) are not serologically related to those of MRDV or PSV. This confirms the result of Milne & Luisoni (1977), who, making the reciprocal test, found that MRDV and PSV antisera did not react with OSDV inner capsids or B spikes.

The OSDV RNA polyacrylamide gel profile confirms that the virus should be placed with the Fijiviruses but, together with the present and previous serological results, indicates that OSDV is a distinct virus within the group. ABDV RNA from both East and West Germany gave bands indistinguishable from those of OSDV, confirming the close relationship found by serology. The present taxonomic position of these and other Fijiviruses is discussed by Boccardo et al. (1979).

Studies of the melting curves and CsCl buoyant densities of available Fijivirus nucleic acids, including those of OSDV extracted from purified SVPs and directly from infected plants, have indicated that all consist of dsRNA; some tRNAs are also present in the preparations (R. I. B. Francki & G. Boccardo, unpublished data). The method used to extract virus nucleic acid directly from infected plants should allow only dsRNAs and tRNAs to remain. DNAs are removed by DNase digestions and ssRNAs are fractionated out with LiCl; in the electrophoretic condition used, the tRNAs migrate off the gels, leaving only the dsRNAs to form the bands observed.

Double-stranded RNA extracted directly from plants was less clean than that from purified SVPs but was about 10 times greater in quantity, enabling us to get RNA profiles of the two ABDV isolates that yielded insufficient SVPs for the test. Similarly, we obtained enough OSDV RNA directly from infected plants to give a profile well before this was eventually possible from purified SVPs. The two methods of RNA extraction also allowed us to compare the directly-extracted ABDV RNA with the OSDV RNA extracted from SVPs.

The OSDV we used had been maintained for 5 years by vegetatively propagating infected plants and the ABDV came from plants similarly propagated for several months. Reoviridae in culture may suffer deletion of genome segments and loss of ability to be transmitted by their vectors (Reddy & Black, 1977; Rubinstein & Harley, 1978). Therefore the transmissibility of OSDV and ABDV by their normal insect vector (see Milne et al. 1975), together with the production of typical symptoms in host plants, provides some reassurance that the virus isolates we tested were genetically intact and representative of field virus.

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**REFERENCES**


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