Symptoms and Electron Microscopy of Ryegrass Mosaic Virus in Different Grass Species

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SUMMARY

A virus from Agrostis stolonifera (tribe: Aveneae) with filamentous particles about 685 nm long was serologically closely related to, and considered to be a strain of, ryegrass mosaic virus (RMV). This strain caused symptoms in Polypogon monspeliensis (tribe: Aveneae) more readily and in Lolium multiflorum (Italian ryegrass) less readily than did RMV from Lolium and Festuca spp. (tribe: Festuceae).

In L. multiflorum and P. monspeliensis the Agrostis isolate induced pinwheel inclusions with associated laminar aggregates and tubes, but Lolium isolates induced pinwheels with laminar aggregates only. The intracellular distribution of the pinwheels differed with the severity of host response. Thus, in plants with mild symptoms most pinwheels were contiguous with the plasmalemma close to plasmodesmata, but in plants with severe symptoms the pinwheels were free in the cytoplasm.

Virus particles occurred either randomly or in bundles in the cytoplasm of mesophyll and phloem companion cells. In P. monspeliensis infected with the Agrostis isolate, fibrous inclusions, possibly virus particles, occurred in some nuclei. When symptoms were severe, mitochondria and chloroplasts were amorphous and the latter had many marginal vesicles.

INTRODUCTION

Ryegrass mosaic virus (RMV), cryptogram R/*:2.7/5:3:E/E:S/Ve/Ac (Slykhuis & Paliwal, 1972) is the most widespread and damaging mechanically transmitted virus of Italian and perennial ryegrasses (Lolium multiflorum and L. perenne) in Britain. It causes a mild to severe, yellow-green streak-mosaic sometimes accompanied by necrotic lesions or a more general necrosis. Severely affected plants of L. multiflorum are dwarfed and even killed. Disease severity varies with the host genotype and/or virus isolate (Wilkins & Catherall, 1974). Particle lengths of different isolates have been variously measured as 703 nm (Brandes, 1964), 675, 703 and 704 nm (Slykhuis & Paliwal, 1972), and 674 or 694 nm (Chamberlain & Catherall, 1977). Like the potyviruses, RMV induces pinwheel inclusions in infected ryegrass (Plumb & James, 1973; Chamberlain, 1974; Paliwal, 1975) and Dactylis glomerata plants (Chamberlain & Catherall, 1977).

Until recently, RMV was thought to occur naturally in Britain only in Lolium, Festuca and Dactylis. In the spring of 1975, we obtained a virus from some Agrostis stolonifera plants having yellow leaf streaks. It reacted strongly with RMV antiserum, but could be

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Table 1. Sources and virulence of virus isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Natural host</th>
<th>Symptoms in natural host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lm₁</td>
<td><em>Lolium multiflorum</em></td>
<td>Mild, streaky mosaic</td>
</tr>
<tr>
<td>Lm₂</td>
<td><em>Lolium multiflorum</em></td>
<td>Severe mosaic, slight necrosis</td>
</tr>
<tr>
<td>Lm₃</td>
<td><em>Lolium multiflorum</em></td>
<td>Severe mosaic, very severe necrosis</td>
</tr>
<tr>
<td>Fp</td>
<td><em>Festuca pratensis</em></td>
<td>Moderately severe mosaic</td>
</tr>
<tr>
<td>Fg</td>
<td><em>Festuca gigantea</em></td>
<td>Mild, streaky mosaic, slight necrosis</td>
</tr>
<tr>
<td>As</td>
<td><em>Agrostis stolonifera</em></td>
<td>Mild, discrete streaks</td>
</tr>
</tbody>
</table>

transmitted to *L. multiflorum* only with difficulty. The present investigations were designed primarily to compare the *Agrostis* virus with some commonly occurring isolates of RMV from *Lolium* and *Festuca*.

**METHODS**

**Virus isolates and host species.** Six locally occurring virus isolates were used. The host species in which each occurred naturally are listed in Table 1. The eight species to which each isolate was inoculated were *Agrostis stolonifera* L., *Avena sativa* L., *Bromus secalinus* L., *Festuca pratensis* Huds., *Lolium multiflorum* Lam., *Phalaris canariensis* L., *Poa annua* L. and *Polygogon monspeliensis* (L.) Desf. Seeds of these species were sown in pots in an insect free glasshouse during April. When the seedlings had from two to six leaves, sap of infected plants extracted in a little neutral 0.1 M-phosphate buffer was rubbed on to them. All inoculated plants were examined periodically for disease symptoms for up to 6 months; no tests for symptomless infection were made.

**Electron microscopy.** Leaves from plants of seven host species/virus isolate combinations differing in symptom severity (see Table 3) were selected for sectioning 5 weeks after inoculation. Leaf pieces (0.5 x 1 mm), yellow at the centre and green at the edges, were cut from systemically infected leaves. Pieces of similar size were cut from comparable healthy plants. Each leaf piece was fixed in 3% (v/v) glutaraldehyde in 0.05 M-neutral phosphate buffer for 3 h at 4 °C. The tissue was post-fixed in 0.2% (w/v) osmium tetroxide in 0.05 M-neutral phosphate buffer containing 0.25 M-sucrose for 2 h at 4 °C, dehydrated through ethanol and embedded in Spurr's low viscosity resin. Sections were cut in an LKB Ultrotome I and stained with uranyl acetate and lead citrate.

Sap containing virus particles was squeezed from the freshly cut ends of infected leaves into 20 μg/ml bacitracin on carbon coated grids, negatively stained in pH 5 sodium phosphotungstic acid, and measured. Magnifications were checked using a standard calibration grating. Sections and virus preparations were examined in an AEI EM6M electron microscope at 80 kV.

**Serology.** Two antisera were prepared. Leaves of *L. multiflorum* infected with isolate Lm₃ or *P. monspeliensis* infected with As isolate were frozen overnight and homogenized in neutral 0.1 M-borate buffer. Homogenates were clarified with chloroform and virus extracted by three cycles of differential centrifugation (2000 g for 20 min; 150000 g for 2 h). Each virus extract (antigen) was injected into a rabbit in four 1 ml portions. Two, emulsified with Freund's complete adjuvant, were injected intramuscularly, and 2 and 3 weeks later the remaining portions were injected intravenously. Blood was collected one week later.

The titres of the antisera were measured using microprecipitin tests, with the antigen at optimum dilution.
Table 2. Number of plants of six species which developed symptoms when inoculated with different isolates of RMV

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Symptoms*</th>
<th>Lm₀</th>
<th>Lm₁</th>
<th>Lm₂</th>
<th>Lm₃</th>
<th>Fp</th>
<th>Fg</th>
<th>As</th>
<th>Total no. plants inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lolium multiflorum (cv. Lemtal)</td>
<td>M</td>
<td>17</td>
<td>12</td>
<td>4</td>
<td>16</td>
<td>12</td>
<td>2</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MN</td>
<td>3</td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Festuca pratensis (cv. Perdita)</td>
<td>M</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>MN</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Bromus secalinus</td>
<td>M</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Avena sativa</td>
<td>M</td>
<td>6</td>
<td>11</td>
<td>12</td>
<td>2</td>
<td>12</td>
<td>0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Poa annua</td>
<td>M</td>
<td>9</td>
<td>15</td>
<td>12</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Phalaris canariensis</td>
<td>M</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

* M = mosaic, MN = mosaic plus necrosis.

Table 3. Relation between symptom severity and the intracellular distribution of virus particles and pinwheel inclusions

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Isolate</th>
<th>Host species</th>
<th>Pinwheel distribution</th>
<th>Virus distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild streak</td>
<td>As</td>
<td>A. stolonifera</td>
<td>Plasmalemma and cytoplasm</td>
<td>Random only</td>
</tr>
<tr>
<td>Mild mosaic</td>
<td>Lm₀</td>
<td>L. multiflorum</td>
<td>Plasmalemma and cytoplasm</td>
<td>Random only</td>
</tr>
<tr>
<td>Mild mosaic</td>
<td>As</td>
<td>L. multiflorum</td>
<td>Plasmalemma and cytoplasm</td>
<td>Random only</td>
</tr>
<tr>
<td>Severe mosaic</td>
<td>Lm₂</td>
<td>P. monspeliensis</td>
<td>Plasmalemma and cytoplasm</td>
<td>Random only</td>
</tr>
<tr>
<td>Severe mosaic</td>
<td>As</td>
<td>P. monspeliensis</td>
<td>Cytoplasm only</td>
<td>Aggregated and random</td>
</tr>
<tr>
<td>Severe mosaic and necrosis</td>
<td>Lm₂</td>
<td>L. multiflorum</td>
<td>Cytoplasm only</td>
<td>Aggregated and random</td>
</tr>
<tr>
<td>Severe mosaic and necrosis</td>
<td>As</td>
<td>L. multiflorum</td>
<td>Cytoplasm only</td>
<td>Aggregated and random</td>
</tr>
</tbody>
</table>

RESULTS

Symptoms and hosts

Different isolates of the virus could be distinguished by the type or severity of symptoms they caused and by their transmissibility to different host species. Some isolates induced necrotic symptoms more frequently than others in Lolium multiflorum and/or Festuca pratensis (Table 2). In Bromus secalinus, isolate Fg produced a severe, Lm₃ a moderately severe and Lm₁, Lm₂ and Fp a mild mosaic; As induced no symptoms. Avena sativa, Poa annua and Phalaris canariensis developed mosaic symptoms of similar intensity to each of the first five isolates but again, gave no symptoms with As. While L. multiflorum and B. secalinus readily developed symptoms with all isolates other than As, F. pratensis, A. sativa, P. annua and P. canariensis did so more frequently with some than with others. Usually it was those isolates most likely to cause necrosis in L. multiflorum, namely Lm₂, Lm₃ and Fg, which were transmitted with greatest frequency. By far the greatest difference occurred between As and the other five isolates, As producing symptoms in a few plants of L. multiflorum cv. Lemtal, but failing to do so in any other species tested. In another experiment As produced symptoms in only one, whereas isolate Lm₃ produced them in all of 100 plants of a single clone of L. multiflorum WPBS Bb 1430. The symptoms in the one As-infected plant of this clone were very much milder than those of any of the Lm₃-infected plants.
The difference between As and the other isolates cannot be attributed wholly to As being a milder isolate with a lower transmission frequency because it was transmitted to 84 out of 95 (88%) of *Polypogon monspeliensis* plants, whereas Lm3 was transmitted to only 6 out of 54 (11%). The symptoms of As and Lm2 in this species were indistinguishable, but those of As took 14 to 20 days to appear, whereas those of Lm3 took 30 to 40 days. Also, As caused symptoms in 2 out of 21 *Agrostis stolonifera* plants inoculated, whilst Lm3 caused symptoms in none.

*Morphology and intracellular distribution of pinwheel inclusions*

Electron microscopy revealed that pinwheel inclusions occurred in the cytoplasm of mesophyll and phloem companion cells of plants of all seven host/isolate combinations examined. Those in Lm1- and Lm2-infected plants were morphologically indistinguishable. They had many laminar aggregates attached to the pinwheel plates, but neither associated scrolls nor tubes occurred. No virus particles were observed between pinwheel plates. By contrast, As-induced pinwheels both in *L. multiflorum* and in *P. monspeliensis* had more distinct plates but with fewer laminar aggregates, and virus particles were often observed between plates and inside the axial tube. Occasionally, associated tubes were present.

The intracellular distribution of the pinwheels was closely associated with symptom severity (Table 3). Thus, pinwheels were mostly continuous with the plasmalemma and close to plasmodesmata in mildly As-infected *L. multiflorum* (Fig. 1), but were always scattered in the cytoplasm abutting on to endoplasmic reticulum in severely As-infected *L. multiflorum*. In the former host/isolate combination, pinwheels were observed only in the centres of yellowed areas of the leaf, but in the latter they were also observed in cells in the surrounding green areas. In *P. monspeliensis*, As induced more pinwheels than Lm3; however, all were scattered in the cytoplasm whereas many of those in Lm3-infected cells occurred at the plasmalemma.

*Distribution of virus particles in infected cells*

Filamentous virus-like particles were observed in the cytoplasm of mesophyll and phloem companion cells of all infected plants examined. No such particles occurred in the cells of healthy plants. In plants with mild symptoms (Table 3) the particles were always scattered throughout the cytoplasm but when symptoms were very severe they often occurred in very large aggregates (Fig. 2). In the three host/isolate combinations where aggregates were observed, some particles occurred in cells in green as well as yellowed areas of the leaf, but in the other four, particles were observed only in cells in the yellow areas. More particles were present in As- than in Lm3-infected *P. monspeliensis*. In the former, virus-like particles sometimes occurred in nuclei (Fig. 3), but in the latter, particles were observed only in the cytoplasm.

In *L. multiflorum* infected with Lm1 and *A. stolonifera* infected with As (both instances where symptoms were mild) micro-inclusion bodies resembling membrane-bound virus aggregates were observed between the cell wall and the plasmalemma (Fig. 4).

*Other ultrastructural changes*

Some changes in cell ultrastructure were common to all host/isolate combinations, but they tended to be greatest where symptoms were severest. Thus, although some abnormal vacuolation and membrane production occurred in the cytoplasm in all instances, it was especially extensive in *L. multiflorum* infected with Lm3 and *P. monspeliensis* infected with As (Fig. 2). With these two combinations, plasmalemmae were frequently withdrawn.
Fig. 1. Section of As-infected *A. stolonifera* cell showing pinwheels in longitudinal section (LS) aligned with plasmodesmata (Pd) and continuous with the plasmalemma (Pm).

Fig. 2. Section of As-infected *P. monspeliensis* cell showing aggregates of virus particles (VP) and cytoplasmic vacuoles (CV). Note association (arrowed) of virus particles with longitudinal (LS) and transverse (TS) sections of pinwheels.
Fig. 3. Section through a nucleus of As-infected *P. monspeliensis* cell showing virus-like particles (VP), and a nuclear extrusion (NE).
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Fig. 4. Section of Lm1-infected L. multiflorum cell showing micro-inclusion body (MIB) between the plasmalemma (Pm) and cell wall (CW), and associated pinwheels.

Fig. 5. Section of Lm1-infected L. multiflorum cell showing irregularly shaped bodies close to the cell wall (CW).

Fig. 6. Section of Lm1-infected L. multiflorum cell showing cell wall deposits at a plasmodesma (Pd).
from the cell wall (Fig. 1); many chloroplasts in infected cells were degenerate with small, marginal vesicles and some chloroplasts had extrusions formed apparently from stromal material. These extrusions sometimes completely surrounded a quantity of cytoplasm occasionally containing a cell organelle such as a mitochondrion. The number of marginal vacuoles and extrusions was greatest in Lm3 infected L. multiflorum and in P. monspeliensis infected with both isolates, where some separation of the granal stacks also occurred. Starch grains in the chloroplasts appeared larger and more numerous in infected than in healthy plants.

Several changes were observed only in specific host/isolate combinations. Usually, nuclei were unaffected, but in As-infected P. monspeliensis they sometimes contained virus-like particles and developed extrusions (Fig. 3). In the cells of plants with mild symptoms, the mitochondria were indistinguishable from those in healthy plants, but in P. monspeliensis and L. multiflorum infected with Lm3 or As, they were amorphous with indistinct cristae. In L. multiflorum infected with Lm1 or Lm3, groups of small, irregularly shaped bodies were observed close to the cell wall (Fig. 5) and cell wall outgrowths occurred, usually near plasmodesmata (Fig. 6).

Serological reactions and particle lengths

Antisera to isolates Lm3 and As had titres of 1:8192 with their homologous antigens but the reaction of Lm3 antiserum with As was stronger (1:496) than that of As antiserum with Lm3 (1:2048). Neither antiserum reacted with extracts from healthy plants.

When 78 particles of each of these two isolates were measured, no statistically significant difference was detected. The modal length of Lm3 from L. multiflorum was 690 nm (s.e. ± 4.79), that of As from P. monspeliensis was 685 nm (s.e. ± 9.94).

DISCUSSION

Wilkins & Catherall (1974) reported that different isolates of RMV differed in frequency of transmission and in the severity of symptoms caused. Similar differences occurred between each of the six isolates studied here. By far the greatest difference was that between As and the other isolates, where a difference in host specificity was accompanied by differences in cell ultrastructure. Thus, in P. monspeliensis, infection with As induced nuclear inclusions similar to those found in Crotalaria spectabilis infected with blackeye cowpea mosaic virus (Edwardson et al. 1972) and in Gloriosa rothschildiana infected with gloriosa stripe mosaic virus (Koenig & Lesemann, 1974), but infection with Lm3 did not. Also, As caused symptoms in fewer species than the other isolates. Nevertheless, on the basis of particle length and serological reaction, we consider As to be a strain of RMV. What may be significant is that As occurred naturally in a genus of the tribe Aveneae whereas all the other isolates occurred naturally in genera of Festuceae. Isolates of phleum mottle virus which occur naturally in Aveneae also cause symptoms in fewer species than isolates from Festuceae (Catherall & Chamberlain, 1977).

Edwardson (1974a, b) suggested that differences in pinwheel morphology could be used to group filamentous plant viruses into three subdivisions; the pinwheel plates may have associated tubes (subdivision I), associated laminar aggregates (subdivision II) or associated tubes and laminar aggregates (subdivision III). Usually, all strains of a virus induce the same type of inclusion. However, the pinwheels induced by RMV isolates Lm1 and Lm3 had many associated laminar aggregates but no tubes (subdivision II), whereas those induced by As had laminar aggregates plus tubes (subdivision III). Another grass-infecting virus which has strains in different subdivisions is sugarcane mosaic virus including the
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related maize dwarf mosaic. Here, too, a strain difference in pinwheel morphology appears to reflect a difference in natural or experimental host specificity (Pirone, 1972; Edwardson, 1974).

Pinwheels are believed to originate at the plasmalemma close to plasmodesmata and later become free in cytoplasm (Langenberg & Schroeder, 1973; Andrews & Shalla, 1974). With RMV the majority of pinwheels appeared to be attached to the plasmalemma in plants with mild symptoms, but were always free in the cytoplasm in plants with severe symptoms. Pinwheels are probably induced by, and their morphology coded for, by the virus because a given virus induces identical inclusions in different hosts (Edwardson, 1974a). Although their precise function is unknown, Chamberlain (1974) found that the level of tolerance of different genotypes of L. multiflorum to infection with RMV was positively correlated with the number of pinwheels present. Pinwheels, therefore, may assist the host to minimize the consequences of infection. Andrews & Shalla (1974) have suggested that if pinwheels do originate at plasmodesmata, their function may be to regulate the intercellular transport of virions and, in so doing, might delay systemic distribution of the virus. In the present work, pinwheels and virus particles were found in green and yellow areas of leaves from severely affected plants, but only in the yellowed areas of leaves from mildly affected plants. This would suggest that rate or efficiency of pinwheel production may be an important factor affecting the severity of the host response to infection.

Micro-inclusion bodies similar to those observed in cells of Lm1-infected L. multiflorum and As-infected A. stolonifera have been reported to occur in L. multiflorum infected with a Canadian isolate of RMV (Paliwal, 1975) and also in various species infected with potyviruses (Lee, 1965; Krass & Ford, 1969; Murant & Roberts, 1971; Barnett, De Zoeten & Gaard, 1971), potato virus X (Allison & Shalla, 1974) and tobacco mosaic virus (Esau & Cronshaw, 1967). They are believed to consist of membrane-bound bundles of virus particles embedded in callose and occur between the cell wall and plasmalemma, usually in close proximity to plasmodesmata and pinwheels. They rarely occur in systemically infected plants and Allison & Shalla (1974) have suggested that they may represent virus immobilized by the host. It is noteworthy therefore that we detected them only when symptoms were mild and that they seem much rarer than in L. multiflorum infected with a mild, Canadian isolate of RMV (Paliwal, 1975).

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REFERENCES


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