Dianthins, ribosome-damaging proteins with anti-viral properties from
*Dianthus caryophyllus* L. (carnation)

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1. Dianthin 30 and dianthin 32, two proteins isolated from the leaves of *Dianthus caryophyllus* (carnation), were purified to homogeneity by chromatography on CM-cellulose. 2. The mol.wt. of dianthin 30 is 29500 and that of dianthin 32 is 31700. Both dianthins are glycoproteins containing mannose. 3. Dianthins inhibit protein synthesis in a lysate of rabbit reticulocytes, with an ID₅₀ (concentration giving 50% inhibition) of 9.15 ng/ml (dianthin 30) and 3.6 ng/ml (dianthin 32). They act by damaging ribosomes in a less-than-equimolar ratio. Protein synthesis by intact cells is partially inhibited by dianthins at a concentration of 100 μg/ml. 4. Dianthins mixed with tobacco-mosaic virus strongly decrease the number of local lesions on leaves of *Nicotiana glutinosa*.

Several plant proteins are known that damage ribosomes, thus inhibiting protein synthesis. Some of them consist of two components, a B-chain capable of binding to cells, and an A-chain that damages the larger subunit of 80S ribosomes. This group includes the toxins ricin, abrin (reviewed by Olsnes & Pihl, 1976), modecinn (Olsnes *et al.*, 1978; Gasperi-Campani *et al.*, 1978) and *Viscum album* (mistletoe) lectin (Stirpe *et al.*, 1980b). Some less-studied lectins that also inhibit protein synthesis probably act in a similar manner, but are not toxic to animals or cells (Barbieri *et al.*, 1979).

Another group of non-toxic protein-synthesis inhibitors are similar to the A-chains of the toxins and includes the pokeweed anti-viral protein (‘PAP’) (Obrig *et al.*, 1973; Irvin, 1975), the wheat-germ inhibitor (Roberts & Stewart, 1979), the *Momordica charantia* (bitter pear melon) inhibitor (Barbieri *et al.*, 1980b) and gelonin (Stirpe *et al.*, 1980a). Both the A-chains of the toxins and the ‘A-chain-like’ proteins (Stirpe *et al.*, 1980a) are thought to be enzymes, since they act catalytically, inactivating a molar excess of ribosomes through an unknown mechanism.

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The pokeweed anti-viral protein, the first of the non-toxic proteins to be purified, was shown to inhibit the replication of tobacco-mosaic virus (Obrig *et al.*, 1973), an effect due to inhibition of protein synthesis in the host cells (Owens *et al.*, 1973). Subsequently it was found that both the toxins and the ‘A-chain-like’ proteins listed above prevented infection by tobacco-mosaic virus. An extract from *Bryonia dioica* (bryony) seeds, which inhibits protein synthesis (Gasperi-Campani *et al.*, 1977), also had anti-viral activity, and an extract from *Dianthus caryophyllus* (carnation) leaves, whose anti-viral activity was known (Van Kammen *et al.*, 1961; Ragetti & Weintraub, 1962a,b), had a strong inhibitory effect on protein synthesis (Stevens *et al.*, 1981).

We report now the purification from *Dianthus caryophyllus* leaves of two proteins, which we propose to name dianthin 30 and dianthin 32 on the basis of their respective molecular weights. Like the known ‘A-chain-like’ proteins, dianthins inhibit protein synthesis in a cell-free system by damaging ribosomes, but have little effect on whole cells. They also have strong inhibitory activity on the replication of tobacco-mosaic virus. Dianthins are probably similar to the two anti-viral proteins purified from carnation leaves by Ragetti & Weintraub (1962a,b).
Experimental

Materials

Leaves of D. caryophyllus were obtained from a local florist and were kept at −30°C if not used immediately. Seeds were purchased from Clause Ltd., Chervil, Reading, Berks., U.K.

L-[14C]Leucine (sp. radioactivity 339–351 Ci/mol) and Na[125I] (16 mCi/μg of iodine) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and L-[14C]phenylalanyl-tRNA (0.35 μCi/mg) was from New England Nuclear Corp., Boston, MA, U.S.A.

CM-cellulose (CM 52) was from Whatman, Maidstone, Kent, U.K.; Sephadex G-25 and G-75 and markers for molecular-weight determinations were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

α-Mannosidase (type III), RNA (type XI), bovine serum albumin and reagents for protein synthesis were from Sigma, Poole, Dorset, U.K. The protein-iodination reagent was from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were of analytical grade.

Ricin was prepared as described by Nicolson & Blaustein (1972) and by Nicolson et al. (1974). A lysate of rabbit reticulocytes was prepared by the method of Allen & Sweet (1962), and rat liver ‘pH 5 supernatant’ as described by Moldave et al. (1971). Ribosomes were prepared from rabbit reticulocyte lysate (Benson et al., 1975) and were resuspended in 80 mM-Tris/HCl buffer, pH 7.4, containing 120 mM-KCl, 7 mM-magnesium acetate and 2 mM-dithiothreitol. Their concentration was calculated from the A260, with the assumptions described by Montanaro et al. (1978).

Tobacco-mosaic virus was purified by the method of Gooding & Herbert (1967). The final A260/A280 ratio was 1.05 (uncorrected for light-scattering).

Samples were diluted in distilled water to give countable numbers of local lesions.

Electrophoresis and determination of molecular weight

Polyacrylamide-gel electrophoresis was performed by using the buffer system of Laemmli (1970), on a discontinuous polyacrylamide gel (upper layer 5% polyacrylamide, lower layer 10% polyacrylamide) at 30 mV for 4 h and at 50 mV for a further 16 h. The following markers were used: bovine serum albumin (mol.wt. 67,000), catalase (60,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000) and soya-bean trypsin inhibitor (20,000). Gels were stained with 0.1% (w/v) Kenacid Blue (BDH Chemicals, Enfield, Middx., U.K.).

Molecular weights were also determined by gel filtration through a column (80 cm × 1.5 cm) of Sephadex G-75, equilibrated with 0.1 M-ammonium formate, pH 8.0, eluted at the rate of 13 ml/h. at room temperature. The following standards were used: bovine serum albumin, ovalbumin, chymotrypsinogen A (mol.wt. 25,000) and ribonuclease A (13,700).

Radioiodination

Dianthins were labelled with 125I by the lactoperoxidase–glucose oxidase method as described in the instructions supplied with the reagent. Labelled proteins contained 1 atom of iodine per molecule.

Protein synthesis

Protein synthesis was determined with a lysate of rabbit reticulocytes and with BL8L cells (Judah et al., 1977) as described by Stirpe et al. (1980b), with the details given in the legends to the appropriate Tables and Figures.

Poly(U)-directed polymerization of phenylalanine was assayed with rabbit reticulocyte ribosomes, essentially as described by Montanaro et al. (1978). Reaction mixtures contained, in a final volume of 0.25 ml: 80 mM-Tris/HCl buffer, pH 7.4, 120 mM-KCl, 7 mM-magnesium acetate, 2 mM-GTP, 200 μg of poly(U), 250 μg (as protein) of ‘pH 5 supernatant’, 20 pCi of [14C] phenylalanyl-tRNA and 20 pmol [calculated as described by Montanaro et al. (1978)] of ribosomes. After incubation at 24°C for 30 min, 0.25 ml of 10% (w/v) trichloroacetic acid was added, and the hot-acid-insoluble radioactivity was determined as described by Montanaro et al. (1978).

Other determinations

Total hexose content was determined by the phenol/H2SO4 method (Ashwell, 1966) and by the anthrone method (Spiro, 1966); galactose was used as a standard because it gives an intermediate colour intensity with the anthrone method.

Protein was determined either by the method of Lowry et al. (1951), with bovine serum albumin as a standard, or spectrophotometrically (Kalb & Bernhohr, 1977).

Ribonuclease and proteinase activity were assayed as described by Razzel (1963) and by Greenberg (1955) respectively, with yeast RNA and with bovine serum albumin as substrates.

14C radioactivity was measured with 10 ml of Insta-Gel (Packard, Downers Grove, IL, U.S.A.) in a Searle Mark III scintillation spectrometer, with an external standard, at an efficiency of approx. 80%.

125I radioactivity was measured in a Packard Auto-Gamma spectrometer.

Antiviral activity

Tobacco-mosaic virus was mixed with the substances to be tested or with an equal volume of water
as a control. Inoculum, containing 600 grit borundum as an abrasive, was rubbed on to leaves of the local lesion host *Nicotiana glutinosa* in a glasshouse at 20–24°C. Each treatment was replicated ten times and randomized on whole leaves of the test plants. Lesions were counted after 3 days of infection.

**Results**

**Purification of dianthins**

A crude extract of *D. caryophyllus* leaves prepared as described below strongly inhibited protein synthesis in a reticulocyte lysate (see Table 1 and Fig. 3 below). The inhibitory factor was purified essentially by the procedure used for the purification of gelonin (Stirpe et al., 1980a).

Carnation leaves (200 g) were washed with distilled water and were homogenized first in a blender and then with an Ultra-Turrax homogenizer, with 320 ml of 0.14 M-NaCl containing 0.005 M-sodium phosphate buffer, pH 7.2. After being stirred overnight at 4°C the homogenate was strained through four layers of cheesecloth. The solid residue was resuspended with 150 ml of medium, and was strained again. The combined extracts were centrifuged at 40 000 g for 30 min, and the supernatant was dialysed against 4 litres of 5 mM-sodium phosphate buffer, pH 6.5, for at least 24 h, with three changes of buffer. The dialysed extract was centrifuged as described above. The supernatant, filtered through Whatman no. 2 filter paper, was applied directly to a column (23 cm × 1.6 cm) of CM-cellulose (CM 52). The column was washed with 200 ml of buffer, and the absorbed material eluted with a 500 ml linear gradient of 0–0.3 M-NaCl in the same buffer. The chromatography was performed at room temperature (22–24°C).

The protein-synthesis-inhibitory activity was recovered with peaks III and IV appearing between 0.1 and 0.2 M-NaCl (Fig. 1). Polyacrylamide-gel electrophoresis showed that these peaks correspond to two single proteins, hereafter called dianthin 30 (peak III) and dianthin 32 (peak IV) on the basis of their respective molecular weights (see below).

The procedure was repeated with consistent results with several batches of leaves. Similar results (not shown) were obtained by extracting leaves that had been dried in the air for 2 months.

Inhibition of protein synthesis was obtained also with extracts of carnation seeds, and purification was attempted with the procedure described above. However, the inhibitory activity was not eluted as a separate peak from the CM-cellulose column, and the procedure was not pursued any further.

The peak identified as dianthin 30 showed a single band in the position corresponding to mol. wt. 29 500 in polyacrylamide-gel electrophoresis after treatment with sodium dodecyl sulphate and mercaptoethanol (Fig. 2). The peak identified as dianthin 32 showed a band of mol. wt. 31 700 and a faint band, not visible in Fig. 2, in the same position as dianthin 30. It should be noted that in the electrophoretic pattern of the crude extract there is a band corresponding to dianthin 32, but no band corresponding to dianthin 30.

Dianthin 30 and dianthin 32 were eluted as single peaks on Sephadex G-75 column chromatography, with mol. wts. 23 000 and 28 000 respectively.

Total neutral-sugar contents, as determined both by the phenol/H₂SO₄ and by the anthrone method, were 2.6% for dianthin 30 and 4.3% for dianthin 32.

![Fig. 1. Purification of dianthins](image)

The dialysed extract (590 ml) from 200 g of carnation leaves was chromatographed on a column of CM-cellulose (CM 52) as described in the text. Fractions (2.2 ml) were eluted with a 0–0.3 M-NaCl gradient (——) at 40 ml/h, and their A₂₈₀ was measured (•). The inhibitor activity on protein synthesis (○) was assayed in the lysate system described in the text with 10 µl of 1 : 1000 dilution of the fractions. Active fractions were pooled as indicated by the horizontal bars.
Radiiodinated dianthin 30 and dianthin 32 bound to a column (4.5 cm x 0.9 cm) of Sepharose–concanavalin A like gelonin (Stirpe et al., 1980a). However, they were not eluted by 0.1M-α-methyl mannose, but could be eluted by 50 mM-sodium borate in 50 mM-Tris/HCl buffer, pH 7.5. After treatment with α-mannosidase, 69% of dianthin 30 and 53% of dianthin 32 did not attach to the column, whereas the rest was bound and could be eluted with borate as described above.

In mice, no toxic effect was observed after intraperitoneal injection of dianthin 32, at doses up to 3 mg/100 g body wt. Dianthins had no ribonuclease or proteinase activity at a concentration of 100 μg/ml.

**Effect on protein synthesis**

*Reticulocyte lysate.* Dianthin 30 and dianthin 32 strongly inhibited protein synthesis in a rabbit reticulocyte lysate, with an ID₅₀ (concentration giving 50% inhibition, calculated by the linear-regression method) of 9.15 ng/ml (95% confidence limits 5.80–14.13) and 3.59 ng/ml (2.31–5.52) respectively, as compared with an ID₅₀ of 1.21 μg/ml (0.54–1.81) of the crude extract (Table 1 and Fig. 3). The recovery of the activity was greater than 100%, a phenomenon observed previously with the inhibitor from *Momordica charantia* (Barbieri et al., 1980b).

The inhibitory activity was unchanged on pre-incubation of the proteins at 37°C for 1 h in the presence of 1% 2-mercaptoethanol, or after freezing and thawing for ten consecutive times, or after keeping at 37°C for 18 h, but was completely abolished by boiling for 20 min. Dianthin 32 retained full activity after freeze-drying; freeze-dried dianthin 30 was poorly soluble and could not be tested. After labelling with ¹²⁵I, dianthin 30 at a concentration of 10 ng/ml inhibited protein synthesis by 41% (unlabelled dianthin, 48%), and dianthin 32 gave a 73% inhibition (unlabelled, 78%).

*Ribosomes.* The toxins and the single-chain proteins mentioned in the introduction inhibit protein synthesis by inactivating ribosomes. Dianthins inhibited phenylalanine polymerization when added to purified rabbit reticulocyte ribosomes (Table 2). A similar effect was observed by treating

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**Table 1. Purification of dianthins**

The purification procedure is described in the text. Data refer to 200 g of carnation leaves. Peak numbers refer to Fig. 1. A unit of activity is defined as the amount giving 50% inhibition of protein synthesis by a rabbit reticulocyte lysate, determined as described in the legend to Fig. 3.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg of protein)</th>
<th>Total activity (units)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysed extract</td>
<td>1292</td>
<td>826</td>
<td>1067000</td>
<td>100</td>
</tr>
<tr>
<td>Dianthin 30</td>
<td>4.37</td>
<td>109300</td>
<td>1067000</td>
<td>115</td>
</tr>
<tr>
<td>(peak III)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dianthin 32</td>
<td>4.39</td>
<td>278000</td>
<td>1223000</td>
<td></td>
</tr>
<tr>
<td>(peak IV)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Purification and properties of dianthins

ribosomes with dianthins and washing them by centrifugation through 5% sucrose before assay. In the latter experiment, dianthin 30 (the only one tested) was effective also at a concentration less than equimolar with ribosomes.

Cells. Dianthin 30 and dianthin 32, at a concentration of 100 μg/ml, inhibited protein synthesis in BL8L epithelial cells, by 61 and 38% respectively (Fig. 4). Ricin, tested for comparison, at a concentration of 10 ng/ml completely inhibited protein synthesis in the same cells.

Anti-viral activity. Dianthin 30 and dianthin 32, mixed with tobacco-mosaic virus before infection, prevented local lesions in the leaves of *Nicotiana glutinosa* by more than 50% at concentrations of 0.5 and 1 μg/ml respectively (Table 3). It should be recalled that in a previous study (Stevens et al., 1981) several purified inhibitors were tested in the same system at a concentration of 50 μg/ml; only

![Graph](image)

**Fig. 3. Effect of dianthins on protein synthesis by a rabbit reticulocyte lysate**

Reaction mixtures contained, in a final volume of 125 μl: 10 mM-Tris/HCl buffer, pH 7.4, 100 mM-ammonium acetate, 2 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-phosphocreatine, 6 μg of creatine kinase, 0.05 mM-amino acids (minus leucine), 0.19 μCi of L-[14C]leucine and 50 μl of a lysate of rabbit reticulocytes. Incubation was at 27°C for 5 min and the radioactivity incorporated into protein was measured in 25 μl samples as described by Gasperi-Campani et al. (1978). Symbols: ○, dianthin 30; ●, dianthin 32; △ (inset), dialysed leaf extract.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Polyphenylalanine polymerized (d.p.m. incorporated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>During preincubation</td>
<td>During assay</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>None</td>
<td>Dianthin 30, 100 pmol</td>
</tr>
<tr>
<td>None</td>
<td>Dianthin 32, 100 pmol</td>
</tr>
<tr>
<td>Dianthin 30, 500 pmol</td>
<td>None</td>
</tr>
<tr>
<td>Dianthin 30, 50 pmol</td>
<td>None</td>
</tr>
<tr>
<td>Dianthin 32, 500 pmol</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2. Effect of dianthins on poly(U)-directed phenylalanine polymerization

Rabbit reticulocyte ribosomes (330 pmol) in 1 ml of 80 mM-Tris/HCl buffer, pH 7.4, containing 120 mM-KCl, 7 mM-magnesium acetate and 2 mM-dithiothreitol, were preincubated at 24°C for 30 min, in the presence or in the absence of dianthin 30 or dianthin 32. Ribosomes were washed by centrifugation at 105 000 g for 3 h through 1.5 ml of 5% sucrose in the same medium. Assays were done as described in the Experimental section with duplicate samples containing 20 pmol of ribosomes.

<table>
<thead>
<tr>
<th>Inhibitor added (μg/ml)</th>
<th>Lesions (mean no.)</th>
<th>Inhibition (%)</th>
<th>Lesions (mean no.)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>60.9</td>
<td>—</td>
<td>80.5</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>22.8</td>
<td>63</td>
<td>54.5</td>
<td>33</td>
</tr>
<tr>
<td>1</td>
<td>5.4</td>
<td>91</td>
<td>12.8</td>
<td>84</td>
</tr>
<tr>
<td>5</td>
<td>2.7</td>
<td>96</td>
<td>11.6</td>
<td>86</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
<td>99</td>
<td>1.9</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 3. Effect of dianthins on local lesion production by tobacco-mosaic virus in leaves of *Nicotiana glutinosa*

Experimental conditions are described in the text.

Discussion

Two proteins, dianthin 30 and dianthin 32, were purified from carnation leaves and appeared homogeneous on polyacrylamide-gel electrophoresis and

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Dianthins seem very similar to the 'A-chain-like' proteins already known from other plants (see the introduction). Thus they, have similar molecular weights, are glycoproteins, appear to contain mannose (like gelonin; Stirpe et al., 1980a), bind to CM-cellulose (like gelonin and the inhibitor from M. charantia; Barbieri et al., 1980b). Like the other proteins, dianthins strongly inhibit protein synthesis in cell-free systems, with little effect on whole cells, and act by damaging ribosomes in a less-than-equimolar ratio.

Dianthin 30 is not visible, on gel electrophoresis, among the proteins of the crude leaf extract, and presumably derives from dianthin 32 or from another protein during the purification by chromatography.

The pokeweed anti-viral protein also exists in two forms (Irvin et al., 1980), and there are several forms of the A-chain of modeccin (Barbieri et al., 1980a), but it is not known whether any of these are present in Nature or appear during the purification procedure.

Both dianthins markedly decrease the production of lesions by tobacco-mosaic virus, and this presumably accounts for the anti-viral properties of carnation-leaf extracts (Van Kammen et al., 1961; Ragetti & Weintraub, 1962a,b), which have been compared with interferon (Fantes & O'Neill, 1964). Our results are consistent with those of Ragetti & Weintraub (1962a,b), who purified from carnation leaves two proteins with anti-viral activity. This is another property that dianthins share with the toxins and the 'A-chain-like' proteins (W. A. Stevens, C. Spurdon, L. J. Onyon & F. Stirpe, unpublished work), and especially with the very potent pokeweed anti-viral protein. The anti-viral activity of the latter was attributed to its inhibitory effect on protein synthesis in the host cells (Owens et al., 1973), and it seems likely that dianthins act through the same mechanism. The fact that dianthin 30 is a less potent inhibitor of protein synthesis in the lysate system is not inconsistent with its greater anti-viral activity. The latter could be due to better penetration into cells, as is suggested also by the greater effect on protein synthesis in whole cells. That the A-chains, and by inference the 'A-chain-like' proteins, may have a different capacity for entering cells is suggested by Uchida et al. (1980), who showed that the A-chain of ricin enters cells more easily than does the A-chain of diphtheria toxin. It is possible that other plant extracts with anti-viral activity contain ribosome-damaging proteins acting in the same way as the pokeweed anti-viral protein and dianthins.

In recent years attempts have been made to bind toxins to appropriate carriers, or their A-chains, to proteins that could act as artificial B-chains, to achieve selective toxicity for specific target cells. [see on gel filtration. Their respective mol.wts. are 29500 and 31700 on gel electrophoresis, and 23000 and 28000 on gel filtration. The difference is not surprising, since the determination of molecular weight of glycoproteins, either by gel filtration (Andrews, 1970) or by polyacrylamide-gel electrophoresis (Segrest & Jackson, 1972), may give erroneous results.

Fig. 4. Effect of dianthins on protein synthesis by BL8L cells Each test was performed on a confluent monolayer of BL8L cells (approx. 2 x 10⁶ cells), growing in a well of an eight-well Multiplate (Lux Scientific Corp., Newbury Park, CA, U.S.A.), in 2 ml of Williams' Medium E (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) supplemented with 5% (v/v) foetal-calf serum, 2 mM-glutamine and gentamycin (50 µg/ml). The appropriate amounts of inhibitors were added dissolved in 10 µl of 5 mM-sodium phosphate buffer, pH 7.2, containing 0.14 M-NaCl. Cells were incubated for 18 h at 37°C in a humidified atmosphere of air/CO₂ (19:1), the medium was then removed and replaced with 2 ml of medium without serum. L-[¹⁴C]Leucine (0.5 µCi/well) was added, and the cells were incubated for a further 1 h before addition of 0.2 ml of 1 M-NaOH to each well. The contents of each well were transferred to test tubes with 1 ml of 0.1 M-NaOH used to wash the well. After 30 min at room temperature protein was precipitated by adding 0.3 ml of trichloroacetic acid (100 g/100 ml) and treated as described by Stirpe et al. (1980b). Symbols: O, dianthin 30; ●, dianthin 32; ■, ricin.
the reviews by Uchida et al. (1980) and Stirpe et al. (1980a). It has been suggested that the non-toxic ‘A-chain-like’ proteins could be advantageously used for such a purpose, and a cytotoxic complex was prepared by binding gelonin to concanavalin A (Stirpe et al., 1980a). Dianthins, and especially dianthin 32, could also be used, with the advantage of being easily prepared from commonly available material.

We thank Dr. K. Cain for gel-filtration experiments.

References
Gooding, G. V. & Herbert, T. T. (1967) Phytopathology 57, 1285