

## Concentration and Purification of Clover Yellow Mosaic Virus from Pea Roots and Leaves

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Journal Paper No. J-7337 of the Iowa Agriculture and Home Economics Experiment Station, Ames. Project 1878.

Accepted for publication 4 February 1973.

### ABSTRACT

Pea roots contained 40-70% as much clover yellow mosaic virus as did tops 10 days after inoculation; the average over all experiments at various times after inoculation was 46%. Roots have lower phenolic content than an equal amount of foliage tissue, and purification

from pea roots eliminates one differential cycle (the difficult chlorophyll removal problem) which helped minimize viral aggregation and loss.

Phytopathology 63:926-930.

Allard in 1916 (2) was probably the first to recover a virus, tobacco mosaic virus (TMV), from roots. Price (13) and Fulton (6) measured the concentration of TMV in roots compared with leaves, by infectivity assays. Stanley (14) purified aucuba mosaic virus protein from roots and found only 25% as much protein from roots as from leaves. Generally roots contain less virus than shoots (6, 14). Mulvania (10) was the first person to inoculate roots of a plant; this was done with TMV.

Some viruses seemingly do not move from infected leaves to roots (3), and some did not move from infected roots to leaves when the roots were

inoculated (10), depending on host-virus combination.

Traditionally, plant viruses are purified from leaves or tops of plants, and many methods have been devised to separate plant viruses from cellular constituents, especially chlorophyll.

I compared clover yellow mosaic virus (CYMV) concentrations in pea roots with its concentration in stems and leaves, and studied the potential advantages of CYMV purification from roots. A preliminary report has been made (4).

**MATERIALS AND METHODS.**—*Pisum sativum* L. 'Perfected Wales' or 'Wilt Resistant Perfection' were

used as host plants for CYMV isolate B, which was obtained from Pratt (11).

*Gomphrena globosa* L., Cornell U. selection (5), was the local lesion host for all infectivity assays. Each value in the virus concentration and purification sections is derived from local lesion counts from 16-24 opposite leaves. The two-dilution assay procedure was used (5).

Tissue for purification was obtained from plants 10-14 days after inoculation, frozen overnight, and thawed. Sap was extracted in the various buffers described in appropriate sections.

Soil was washed from pea roots in several early experiments to determine virus concentration. Roots are more readily recovered from a Perlite medium so that was used in all succeeding experiments. Seedlings were transplanted to individual boxes containing nutrient (Hoagland's) solution (7) 6-7 days after planting. This provided easy accessibility to roots for inoculation, observation, and harvest for purification. Virus inoculations were made 2-3 days after transplanting by rubbing diluted infective solutions on 400-mesh, Carborundum-dusted leaves or roots. Plants were harvested 10-14 days after inoculation.

Purification was at temperatures ranging 0-3 C. Low-speed centrifugations, were in a Sorvall SP/X centrifuge in a 1 C cold room, and high-speed centrifugations were in a Spinco model L, refrigerated ultracentrifuge fitted with a No. 30 or 40 preparative rotor. The relative virus loss at each stage of purification was assessed by assaying all supernatant and pellet materials on *G. globosa* L. and converting the values for comparison with starting tissue weights. More highly purified preparations were monitored also by spectrophotometric measurements in a Beckman DB spectrophotometer, then converted to protein content. The protein conversions were made by taking the 260 and 280 nm readings from the spectrophotometer, locating them with the aid of a straight edge on the Nomograph (by E. Adams, distributed by California Corporation for Biochemical Research, 3625 Medford St., Los Angeles, Calif.), and reading the protein content directly.

Purification treatment for each experiment always included a parallel batch of tops and roots from the same plants. A typical purification experiment with minor variations, involved five steps. (i) Tissue was harvested and ground with an equal volume of buffer (w/v). The brei was removed by squeezing through gauze. The two variations during the clarification process were, either freezing the tissue overnight before grinding, or immediately adjusting the sap to pH 5.4 with 10% acetic acid after removing the brei and letting it set for 1 hr at 24 C. (ii-v) The supernatant was centrifuged at alternating low (10,000 g for 10 min) and high (ca. 90,000 g for 90 min) speeds. Viral pellets were suspended in successively smaller amounts of buffer. Infectivity assay dilutions of each step were based on original volume of tissue extract.

Sedimentation coefficients were determined for CYMV obtained from the final alternate cycle by centrifuging the purified CYMV in either 0.01M

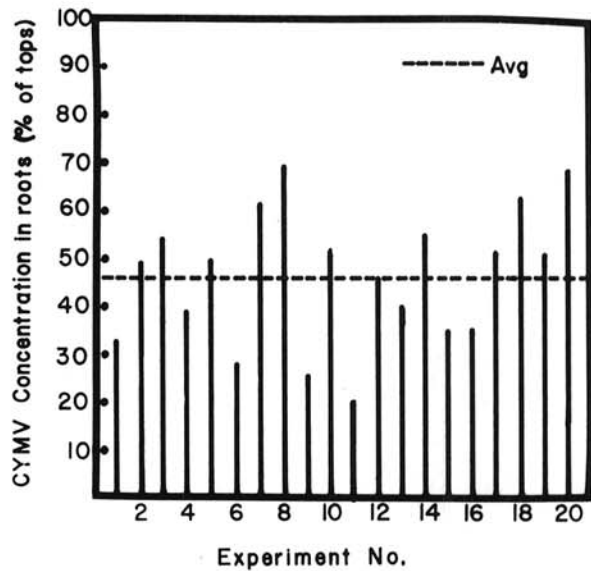


Fig. 1. A summary of 20 tests comparing clover yellow mosaic virus (CYMV) infectivity in roots versus tops (w/w) of peas.

dipotassium phosphate, pH 7.0, or 0.05M Na borate, pH 8.4, in an AnD rotor in a Spinco model E, analytical ultracentrifuge with Schlieren optics. The sedimentation coefficients were calculated for 20 C in water.

**RESULTS.—Virus concentration.**—The first three experiments involved a measurement of CYMV concentration in (i) roots, (ii) stems, including pedicels and tendrils, and (iii) leaves, including stipules, from leaf-inoculated plants. CYMV infectivity from leaves was chosen as the reference standard, 100%. There was less relative infectivity in roots (60%) than in stems (82%) or leaves. No visual difference was apparent between roots of CYMV-infected plants and roots of noninoculated plants. All subsequent experiments compared roots with tops, which included all above-seed tissue.

Relative CYMV concentration in roots compared to tops varied both with plant age and season. Percentages were 34% in April, 5 weeks after inoculation; 69% and 53% in June, 8 and 11 days after inoculation, respectively; and 1, 4, 1 and 16% in August, 4, 7, 9, and 11 days after inoculation, respectively. All subsequent tests, 10-25 days after inoculation, showed an over-all average of 46% as much CYMV in roots as in shoots (Fig. 1).

**Root vs. top inoculation.**—An experiment was done to determine the effect of route of virus entry on symptom appearance and CYMV concentration in root and top tissue and on root condition. Group 1 was inoculated by removing the pea plants from the Hoagland's solution, dusting the roots with Carborundum, and rubbing with inoculum between thumb and forefinger. Group 2 was inoculated on leaves, and inoculum was added to the nutrient medium of group 3 without disturbing the roots.

CYMV concentration in roots is compared in Fig. 2 for root- vs. top-inoculated plants. No symptoms appeared in the plants in group 3. Roots and tops of 10 plants were selected at random from group 3, rinsed, and assayed on *G. globosa*. No local lesions developed.

Three plants of the 24 in group 2 had symptomless leaves at the end of the experiment. The roots and tops of these three peas were assayed; CYMV was present in the roots, but not in the tops.

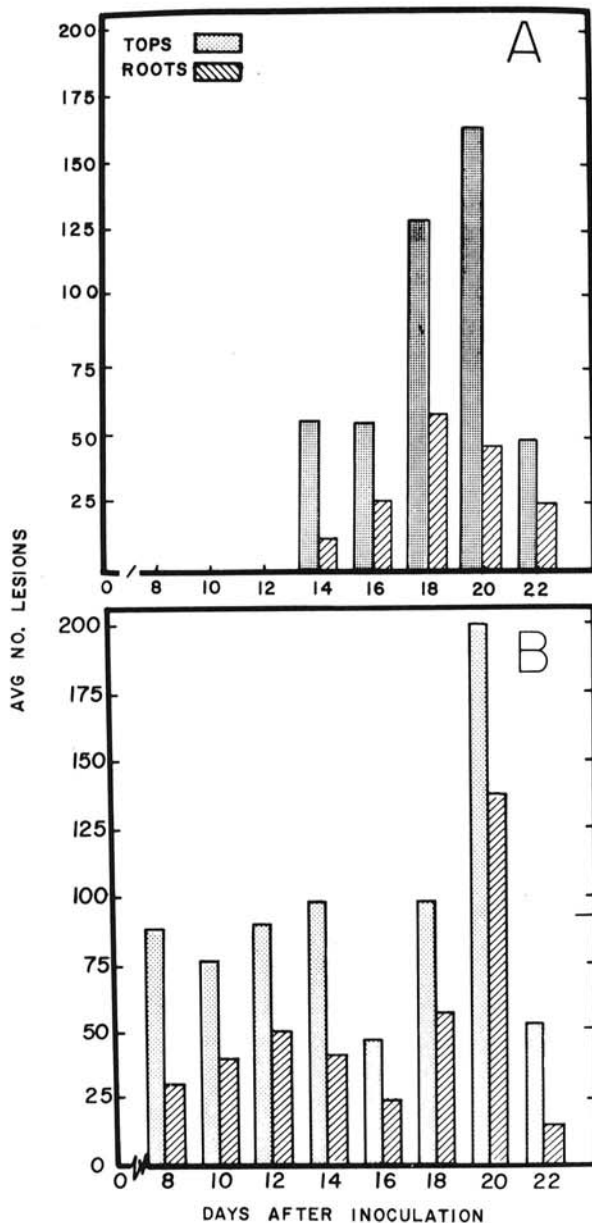


Fig. 2. Relative concentration of clover yellow mosaic virus (CYMV) in roots and tops of peas following A) root inoculation and B) top inoculation. Local lesion assays were made on 20 opposite leaves of *Gomphrena globosa* for each test.

TABLE 1. Infectivity of clover yellow mosaic virus (CYMV) purified by differential centrifugation from pea roots and tops ground and processed in three different systems (distilled water, 0.01M neutral phosphate buffer, or 0.05M borate buffer, pH 8.4)

Centrifugation cycle	Treatment					
	Borate		Phosphate		Water	
	Root	Top	Root	Top	Root	Top
A <sup>a</sup>	55	100	51	100	51	100
B	47	20	84	63	96	84
C	26	25	28	88	30	74
D	42	47	82	95	128	63

<sup>a</sup>The expressed sap from tops clarified by low-speed centrifugation was used as the 100% infectivity basis. Subsequent high-speed, low-speed differential centrifugation cycles were assayed after the initial low-speed cycle (B, C, and D).

CYMV moved more rapidly from tops to roots and reached higher concentrations in both roots and tops than from roots to tops (Fig. 2).

*Purification.*—Infective, clarified root sap appeared clearer (and never as dark brown) than top sap during infectivity assays. Thus, purification from roots was viewed as possibly more advantageous than from tops because of the fewer normal plant components and discoloring agents.

Almost 10% less sap was obtained from tops than roots, although fresh weight of tops is 35% greater than that of roots. The pH of root sap was usually about 0.3 pH unit lower than that of top sap.

The amount of infectivity retained through a purification scheme of four cycles (Table 1, A through D) of differential centrifugation was generally as great or greater from roots than from tops, regardless of buffer used. Data from clarified juice (Table 1-A) is based on 100% infectivity from tops.

The virus pellets from H<sub>2</sub>O or borate-buffer purification cycles were very clear compared with those from a phosphate buffer system (Fig. 3).

The average losses are 6% in the sedimentable material during clarification via the original low-speed centrifugation. All values are calculated back to the infectivity of the initial clarified sap. The supernatants following 100,000-g centrifugations for 60-90 min contained 0.3% of the infectivity. Thus, these accounted for only 1-1.5% loss during a purification schedule. Greatest losses occurred during low-speed clarification, where 0.9-1.5% loss occurred each time in the sedimentable debris. CYMV was most likely absorbed to or mixed with these sedimentable materials. The expected mechanical losses amounted to about 6-15% during a complete procedure; thus, any greater losses could be accounted for by virus inactivation or aggregation, measured directly by lack of infectivity.

*Infectivity assays and spectrophotometer measurements.*—The virus concentration at each step of the purifications was measured spectrophotometrically and with infectivity assays.

Phosphate-purified preparations had consistently lower O.D. readings than water-purified preparations, even though infectivity tended to be nearly the same or higher in phosphate-buffer preparations (Fig. 4).

*Electron microscopy.*—No size difference of CYMV particles was noted, whether the source was roots or tops. Examination of virus particles after purification in various buffers, indicated that severe aggregation was noticeable in phosphate, and many fragments were observed in borate buffer where little aggregation was seen.

*Sedimentation.*—The purified CYMV from pea tops was centrifuged in a Beckman model E analytical centrifuge with Schlieren optics to determine sedimentation coefficients ( $S_{20,w}$ ). There were typically two or three peaks in the sedimentation patterns. Their values were 188 S, 203 S, and 282 S in 0.01M neutral phosphate buffer and 135 S, 182 S, plus an occasional peak near 250 S in 0.05M Na borate buffer, pH 8.4. The value of 135 S compares with the value of 121 S published previously (12).

**DISCUSSION.**—Pea roots support only about half as much CYMV as tops. CYMV titer in roots and tops may vary with the seasons. It was lowest in summer. This probably relates directly to temperatures above 30 C, not uncommon in greenhouses in the summer. McLean (8) inoculated roots of many plants with tobacco ringspot virus. He found that soil temperature markedly influenced virus movement from the roots. Some viruses never move from the roots to the tops (10). CYMV did not move from roots in a few pea plants, although the virus did multiply there. I did not determine why CYMV did not move into the stems and leaves of these few plants.

CYMV replication starts more slowly following root inoculation, and CYMV is undetectable in both tops and roots until the 14th day (Fig. 2); CYMV concentration was never as high in either roots or tops compared to that after top inoculation. The tissues of organs where virus entry occurs may have a marked influence on the ultimate virus concentration throughout the plant. Only half as much CYMV was present in roots as in tops, regardless of mode of entry.

Sap from pea roots never turned as dark brown as that from pea tops, suggesting the presence of a weaker phenol oxidase system in roots, or at least a smaller amount of oxidizable substrate. There may be weaker inhibitors or inactivators, which result in greater relative infectivity from roots after purification. Fulton (6), however, speculated that inhibitors in roots prevented concentrations of viruses in roots as high as in tops.

The spectrophotometric measurements combined with infectivity assays seemed to be closely correlated and were generally useful, showing that phosphate buffer helped to maintain infectivity better than water.

One explanation of more than 100% infectivity in late stages of purification, or, a higher infectivity with succeeding differential cycles, is viral aggregation at

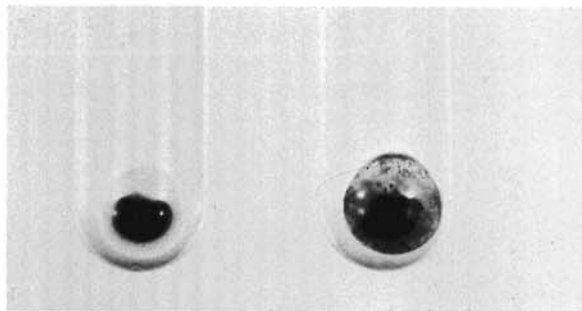


Fig. 3. Clover yellow mosaic virus (CYMV) pellets from root tissue in centrifuge tubes after a second high-speed cycle. The dark central eye is disposable nonvirus material, the transparent overlay material is infective from H<sub>2</sub>O (borate buffer was comparable) on left and from PO<sub>4</sub> buffer on right.

certain stages, making it difficult to accurately assay infectivity.

There is an actual "gain" in amount of virus, based on infectivity, purified from roots compared with tops, partly because one cycle of differential centrifugation can be eliminated, and partly because less aggregation and phenolic inhibition may occur.

It is not popular to study roots of virus-infected plants. The main advantage in purifying virus from roots is elimination of the difficult chlorophyll-removal problem. Watson & Guthrie (15) studied roots of red clover carefully, and determined that some root rots are caused by virus infections.

Pratt & Reichmann (12) say that mild acidity tends to aggregate CYMV. Even though the pH of sap from infected roots of peas is normally 0.3 unit lower than the pH of sap from tops, this more acidic

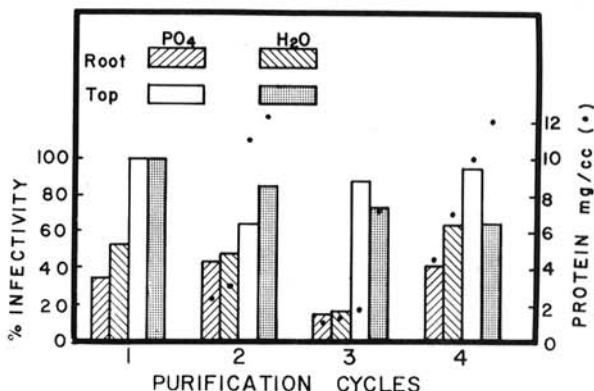


Fig. 4. Protein and infectivity measurements from a typical purification in 0.01M dipotassium phosphate buffer, pH 7.0 (PO<sub>4</sub>) or demineralized distilled water (H<sub>2</sub>O) of clover yellow mosaic virus (CYMV) from pea roots and tops. Bars grouped by cycles 1-4 represent the result of each successive alternate low-high speed centrifugation cycle. Infectivity was assayed on *Gomphrena globosa*. Protein determinations were converted from O.D. readings taken with a Beckman DB spectrophotometer.

environment did not seem to cause noticeable aggregation.

The aggregation of CYMV particles in phosphate buffer, seen by electron microscope examination, was reflected in higher  $S_{20,w}$  values. Some aggregation also occurred in the borate buffer because two and sometimes three sedimentation peaks were seen in that preparation. Agrawal et al. (1) claim to have obtained a single distinct peak when they purified in 0.1M Na phosphate buffer, pH 7.0, but I was unable to repeat that work. I always got three distinct peaks.

This study led to a more detailed examination of viruses in roots. Viral concentrations in roots will vary with host-virus combinations (9). In certain instances where difficulty is encountered in virus purification, it would seem advantageous to try purifying from roots.

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