Spring 1964

# RELATION OF STEM PITTING OF APPLE TO OTHER APPLE VIRUS DISEASES

RICHARD CASWELL MC CRUM

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BY

RICHARD C. MC CRUM

B. S., University of Arizona, 1951

M. S., University of Maine, 1953

A DISSERTATION

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Botany

May, 1964

This dissertation has been examined and approved.

Avery Rick

Stuart Dunn

Albion R. Hodgdon

T. V. Moore

J. G. Conklin

May 21, 1964
Date

## ACKNOWLEDGMENTS

The author is indebted to many people for assistance during the course of this work and particularly to Dr. A. E. Rich for encouragement in the continuance of the work and for guidance in the manuscript preparation. He also wishes to thank Dr. T. G. Metcalf for his valuable suggestions and for the use of special equipment and serological supplies, Dr. S. C. Smith for cooperation and use of the ultracentrifuge, and Dr. W. C. Skoglund for providing quarters for the rabbits used in the serological studies.

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# SECTION I

## INTRODUCTION

Severe winter injury due to low temperatures has plagued commercial apple growers periodically and has caused failures with many attempts to extend the range of commercial apple growing in the Northeast and into certain midwestern locations. Major damage to apple trees occurs when trunks and scaffold branches are injured. To prevent this damage several "cold resistant" bodystocks have been used to form the framework upon which desired scion varieties can be top-worked. The variety Virginia Crab was used as a hardy bodystock in the 1930's and '40's, and reports from Iowa during this period clearly indicate the benefits of selecting this clone as a "cold resistant" variety. However, later attempts to duplicate the success of Virginia Crab as a hardy stock in other apple growing areas met with failure. The same combination of stock and scion varieties found compatible in Iowa tests were, in general, unsuccessful in later trials although some superior combinations resulted. It is now known that failure to duplicate results in later attempts with Virginia Crab as a bodystock was due to latent virus infection occurring in the scion varieties to which Virginia Crab was sensitive.

The general syndrome characterizing virus infection in Virginia Crab begins with the formation of abnormal vascular tissues, eventually resulting in a macroscopic symptom

known as stem pitting (Fig. 1). Tree decline gradually takes place due to the disruption in conducting tissues. Many plantings employing Virginia Crab as a hardy stock have since been removed with considerable loss in revenue to the apple industry. These losses have led to a reluctance of growers to use hardy stock scion combinations in areas where periodic occurrences of severe winter killing temperatures can be expected. It has also resulted in an interruption of testing hardy apple stocks until a re-evaluation of existing apple stocks is made in order to find material free from latent apple viruses.

The present work was undertaken to compare the virus entity causing stem pitting in Virginia Crab with other latent apple virus factors and to attempt to find clones of apples free from stem pitting and other latent virus entities. Information obtained from such studies, it is hoped, would renew an interest in the use of hardy framework varieties of apple and would furnish material suitable to study the effect of the latent stem pitting virus on yields and tree growth in tolerant varieties.

Figure 1. Stem pitting symptoms on a Virginia Crab apple trunk top-worked to Baldwin. Note only three seedling roots remain to support the tree.

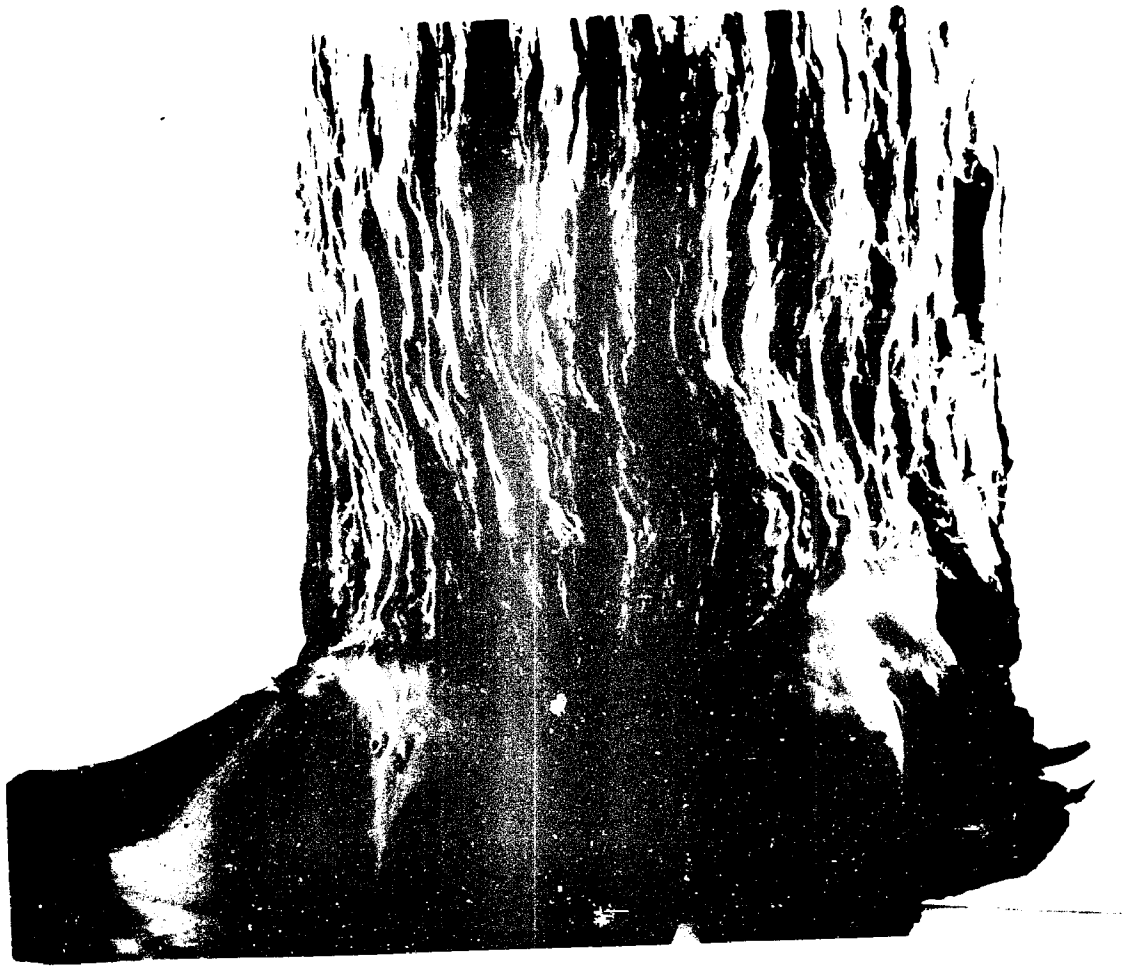

Figure 1

## SECTION II

## REVIEW OF LITERATURE

The term "stem pitting" was adopted by Smith (1954) to describe a disorder occurring on Virginia and Florence Crab apple varieties used as bodystocks in New Hampshire. This name seemed appropriate because of a similarity in symptoms to a stem pitting described by Bitters and Parker (1953) on quick decline of citrus. Symptoms of the stem pitting in apple were clearly described and depicted by Smith (1954), and he suggested that a virus might be the causal agent. In the same year Tukey et al. (1954) described similar wood pitting in Virginia Crab bodystocks and they noted the disorder occurring in Virginia Crab resembled the tristeza disease of citrus. They suggested that possibly this uncongeniality shown by Virginia Crab to several scion varieties was due to a virus that had "crept into certain varieties of apples" and that if true then Virginia Crab might serve as a "useful indicator plant for certain latent apple viruses".

Prior to 1954 several articles contained reports of the uncongeniality of Virginia Crab when top-worked to certain scion varieties. Lantz (1933) first reported Virginia Crab to be uncongenial with other fruit varieties. Maney et al. (1936) pointed out that the apple variety Willow Twig produced only one-half the growth on Virginia Crab that it produced on Hibernal, another hardy stock variety. McClintock (1938)

listed several incompatibilities occurring with Virginia Crab top-worked to fruiting scion varieties. He found that the combination of Blaxtaman and Virginia Crab indicated a definite lack of affinity. Tree growth in the latter case was rated at 17% compared with Golden Delicious which was completely compatible with Virginia Crab.

It has since been found that small-fruited, crab-apple type varieties are generally more sensitive to common latent viruses such as stem pitting than are the large fruited apples.

Many reports of incompatibilities between apple varieties can be found in the literature. Production of dwarf types of flowering crab has, in the past, met with failure. Undoubtedly, as pointed out by Campbell (1962), many of the failures were due to the presence of latent virus infection occurring in clonally propagated dwarfing root-stocks to which the crabs were grafted. Aichele (1961) listed several Malling rootstocks as carrying stem pitting virus, and many of the 58 varieties of flowering crab used by him as indicators reacted to rootstocks infected with stem pitting. Reynolds and Milbrath (1961) found numerous crab apple varieties to be useful as quick indicator hosts for latent apple virus factors.

Transmission and identification of the virus entity causing pitting in Virginia Crab is complicated by the fact that more than one latent virus may be present in apple material used as inoculum. Millikan and Guengerich (1954) found that buds from trees of Virginia Crab infected with

bark splitting disease grafted to Prunus tomentosa produced a striking leaf mottle on the latter indicator and thus showed that an infectious entity was responsible for bark splitting in Virginia Crab. Later tests with similar diseased material produced definite leaf symptoms on Amelanchier sp. (Millikan and Guengerich, 1956). Confirmation of the viral cause of stem pitting of Virginia Crab was not obtained, however, until Guengerich and Millikan (1956) actually transmitted the stem pitting virus factor in grafting experiments with buds and bark from diseased, pitted, trees to non-pitted Virginia Crab. Classification of the stem pitting factor of apples and its relationship to other latent viruses is at present based on symptom expression in indicator host studies. McCrum (1962a) pointed out the danger of identifying causal agents of apple virus diseases based on similar host symptom expressions occurring in heterogeneous Malus sp. He found that virus isolates which did not produce stem pitting symptoms in Virginia Crab did cause wood pitting in the sensitive Russian Crab R-12740-7A. These differences in host reaction signified that separate strains of stem pitting virus existed or that similar symptoms were produced on different hosts by distinctly different viruses.

Stem pitting, however, is a general reaction symptom characteristic of several diseases of woody plants, such as quick decline of citrus (Bitters and Parker, 1953), and stem pitting of coffee (Sheffield, 1959), which may or may not have a definite virus origin. Mink and Shay (1962) point

out the occurrence of a wood pitting disorder non-transmissible to Virginia Crab which was found in apple seedlings that had never been worked to other varieties. Triploid seedlings from 4N x 2N crosses showed the greatest incidence and severity of pitting. Apple stem pitting has been found to occur separately or in conjunction with other apple latent viruses when individual trees have been sampled. Cation and Carlson (1960) made a survey of latent virus content in a Michigan apple scion rootstock test orchard and found that there was no definite pattern of virus content in trees sampled. Indexing tests run on 28 trees which gave a positive reaction for either stem pitting or chlorotic leaf spot revealed that 14.3% contained the chlorotic leaf spot entity minus stem pitting, while 21.4% contained only stem pitting without giving a positive reaction for chlorotic leaf spot on R-12740-7A. From such information they concluded that stem pitting and chlorotic leaf spot appeared to be caused by different viruses. Cation and Carlson (1962), in further studies, again produced data confirming that stem pitting and chlorotic leaf spot reactions could serve to differentiate different virus entities. They found that the sensitive apple clone R-12740-7A did not react to isolates known to contain only the stem pitting factor. In addition, the stem pitting factor alone did not cause reaction patterns in Floribunda and Elyii Crabs, nor did dieback occur on the indicator Spy 227. They pointed out that since negative readings occurred on the R-12740-7A indi-

cator with the latter isolate, this host was unsatisfactory as an indicator for the stem pitting virus. They suggested that Spy 227 should be used in addition to R-12740-7A when indexing for apples for latent viruses containing stem pitting. Cation and Carlson (1962) also found that variations in stem pitting symptoms occurred, implying that different strains of stem pitting factors existed.

Shay and Mink (1961), who were the first to report the disease syndrome known as chlorotic leaf spot, found that trees with severely pitted Virginia Crab interstocks frequently gave a positive reaction for both chlorotic leaf spot and stem pitting. Several trees, however, with non-pitted Virginia Crab interstocks indexed positive for chlorotic leaf spot. Thus they concluded that chlorotic leaf spot of R-12740-7A and stem pitting of Virginia Crab are disease reactions due to distinct viruses which may occur separately or together as latent forms in commercial apple varieties. The latter authors also revealed a widespread occurrence of the chlorotic leaf spot factor in commercial apple varieties and clonal rootstocks. That such widespread infection of chlorotic leaf spot occurs would tend to explain why the 2 entities, chlorotic leaf spot and stem pitting, are often confused in attempts to classify the causal agents when single or non-specific apple indicators are employed.

Posnette and Cropley (1961) indicate the problematic and relative merits of using separate indicator hosts for

different virus isolates. In testing a range of Malling-Merton and Malling rootstocks, as well as 3 scion varieties, they found that some clones produced leaf, bark, and xylem symptoms in the indicator clone Spy 227 and Malus platycarpa, while others produced only leaf symptoms on the latter hosts. Some isolates tested produced stem pitting on Virginia Crab in addition to reactions on Spy 227 and Malus platycarpa. The isolates which pitted Virginia Crab also caused a green mottle when grafted to peach seedlings. These findings were substantiated by Posnette and Cropley (1963). Reciprocal tests back to apple have not been reported.

Sap transmission of viruses from apple isolates to Chenopodium quinoa has been obtained with material that indexed positive for chlorotic leaf spot but which was free of stem pitting as well as for isolates containing both virus factors (Cropley, 1963). In the successful sap transmission tests two of the isolates which did not contain stem pitting, as indexed on Virginia Crab, also did not cause decline when inoculated into Spy 227. No tests, however, were made with isolates containing the stem pitting factor as a single entity. Isolates tested in the above study also gave consistent readings in graft-inoculation tests with apple, pear, and quince, indicating that the causal agents of apple chlorotic leaf spot, pear ring spot, and line mosaic appeared to be identical. Cadman (1963) also isolated a virus from apples containing chlorotic leaf spot only and found it to be similar serologically to a Prunus

pissardii strain of raspberry yellow dwarf virus. He suggested that the above mentioned virus bore some physical resemblance to sugar beet yellows virus, although he indicated that evidence of association should not constitute proof of causal relationship.

Dwarf-fruit and tree-decline of Hyslop Crab is reported by Cation (1960) to result from infection with a virus similar to other viruses that produce apple stem pitting, signifying a definite relationship between this disease and that of stem pitting.

Virus classification based on interpretation made from host range studies is complicated by the fact that more than one latent virus may occur in the initial inoculum source tested as well as in the indicator hosts themselves. Stem pitting has been found to exist in a latent form in the rubbery wood indicator Lord Lambourne by Posnette and Cropley (1961). Since it did not produce symptoms in the latter indicator it does not appear to be related to the causal agent of rubbery wood. Welsh and Keane (1959) also present evidence to show that the causal agents of rubbery wood and stem pitting of apple are distinct viruses. They found that although stem pitting and rubbery wood could occur together in apple, 1 clone of Golden Delicious which was top-worked to a non-pitted Virginia Crab was found which indexed positive for the rubbery wood factor when this clone was grafted to Lord Lambourne. Cation (1961) reported that neither dwarf-fruit isolates nor those containing Spy 227-

lethal could be made to produce mosaic or rubbery wood symptoms in Lord Lambourne in grafting tests conducted over a 2-year period. Since the isolates used by Cation were "stem pitting" factors, this would offer further evidence that the rubbery wood causal agent is not related to the stem pitting virus. Several strains of rubbery wood, when grafted to Virginia Crab, have produced no obvious symptoms 3 years after inoculation, according to Welsh and Keane (1961). In another paper by the same authors (Keane and Welsh, 1961), they again point out that rubbery wood and stem pitting can occur independently of each other in indexing tests. Furthermore, they found that more than 200 Virginia Crab trees showed no stem pitting symptoms when propagated on Malling II rootstocks which had given a positive reaction to chlorotic leaf spot. McCrum (1962) found that stem pitting isolates did not cause rubbery wood nor produce mosaic symptoms in Lord Lambourne 2 years after initial budding. Mink and Shay (1962) indexed several clones known to contain chlorotic leaf spot and stem pitting on Lord Lambourne for rubbery wood, but only negative results were obtained. In fact, 2 of the Lord Lambourne clones used as indicators indexed positive for both chlorotic leaf spot and stem pitting, apparently occurring as latent infections since no symptoms were evident on Lord Lambourne.

Mink and Shay (1962) point out the dangers of adopting a limited number of existing indicator plants for routine indexing and suggest that as the list of latent viruses

in apples continues to enlarge, the number appears to be limited only by the number of indicators used and isolates tested. It should be noted that a standard minimum range of indicators listed for apple tree viruses in Europe (Posnette, 1963) includes Virginia Crab as one of the required indicators.

# SECTION III

## MATERIALS AND METHODS

### Virus Isolates

Stem pitting: Originated from the framework portion of a pitted Virginia Crab tree top-worked to Baldwin located in a hardy stock planting at Highmoor Farm, Maine.

Dapple apple: Obtained from W. W. Smith, Department of Horticulture, University of New Hampshire, Durham, New Hampshire. The budwood was taken from a Cortland apple tree exhibiting dapple apple symptoms top-worked to a non-pitted Virginia Crab.

Flat limb: Obtained from Kentville, Nova Scotia. Consisted of Gravenstein budwood from an experimental plot of flat limb infected trees.

Cortland mosaic: Consisted of budwood taken from a Cortland apple tree in an orchard at Kent's Hill, Maine.

Russet ring: Obtained from E. L. Reeves, Wenatchee, Washington. It consists of budwood from Golden Delicious tree X-39T, AP-3 exhibiting russet ring symptoms.

Green mottle: Obtained from D. H. Palmiter, Hudson Valley, New York. Consists of budwood from an infected Duchess of Oldenburg tree #1-4 that has shown characteristic symptoms of green mottle each year.

1-3 B: Obtained from D. Cation, East Lansing, Michigan. This apple isolate contains stem pitting but does not produce symptoms on the indicator R-12740-7A (Cation, 1962).

Granny Smith PI 88571: Originally obtained from Glenn Dale, Maryland, for use as an indicator; subsequently found to contain several virus factors.

Dwarf fruit: Obtained from D. Cation, East Lansing, Michigan.

Quince mosaic: Collected from a naturally infected ornamental quince (Chaenomeles sp.) located at Highmoor Farm, Maine.

Quince stem pitting: A seedling quince inoculated with the stem pitting isolate at Highmoor Farm, Maine.

Quince 9-23: A quince seedling inoculated with a non-pitted Virginia Crab source tree 9-23.

Chlorotic leaf spot: Obtained from G. I. Mink, formerly at Purdue University, Lafayette, Indiana. Consists of a non-pitted Virginia Crab clone C-8 which does not contain stem pitting but produces reactions on R-12740-7A, Spy 227, and Malus platycarpa (Long Ashton strain).

Rubbery wood: Obtained from R. M. Gilmer, New York Agricultural Experiment Station, Geneva, New York. Consists of budwood of Lord Lambourne apple showing rubbery wood, originally on EM I rootstock.

Erb mosaic: Collected by Andrew Phillips, Acton, Massachusetts, from a tree in the Erb apple orchard, Hudson, New Hampshire. The original tree variety is not known.

1-12 A: Obtained from D. Cation, East Lansing, Michigan. This isolate came from a Red Delicious O-524/Bedford tree in an apple scion rootstock test orchard. As indexed by D. Cation (1962) this isolate does not contain the

chlorotic leaf spot factor, indexes negative on Floribunda and Elyii Crabs, and contains stem pitting as well as producing dieback and internal bark necrosis in Spy 227.

LM-33 (Spy Lethal): Obtained from D. Cation, East Lansing, Michigan.

Cortland J-5 ):
Cortland I-12 )
Cortland J-30 )
Cortland E-27 ) These 4 isolates were obtained from the New Hampshire orchard used by J. Barrat (1958) in his studies on dapple apple.

Scar skin: This isolate was found originally on a Red Delicious-Hibernal tree in a planting of the Berry Hill Orchards, Maine.

Apple Indicators

Cox's Orange PI 247021: Blangsted or Kortegaard's strain. It was received from Plant Introduction Station, Glenn Dale, Maryland.

Belle de Boskoop PI 199684: Obtained from Plant Introduction Station, Glenn Dale, Maryland. It is a red strain known as Jensen's Red Strain or Bogo Strain.

Sugar Crab PI 143974: Obtained from Plant Introduction Station, Glenn Dale, Maryland.

R-12740-7A: Obtained from G. I. Mink, formerly at Purdue University, Lafayette, Indiana. Free of any known latent viruses.

Malus platycarpa: Obtained from R. M. Gilmer, New York Agricultural Experiment Station, Geneva, New York. This apple clone originally came from the Long Ashton Station,

England, and has been designated as M. platycarpa (Long Ashton strain).

Malus sieboldi arborescens: Seedlings from seed obtained from F. W. Schumacher, Horticulturist, Sandwich, Massachusetts.

Malus floribunda: Seedlings from seed obtained from F. W. Schumacher, Horticulturist, Sandwich, Massachusetts.

Lord Lambourne PI 238789: Obtained from Plant Introduction Station, Glenn Dale, Maryland.

Lord Lambourne (Geneva): Obtained from R. M. Gilmer, New York Agricultural Experiment Station, Geneva, New York. This clone indexes negative for stem pitting and chlorotic leaf spot factors.

Quince: Seedlings from seed of an ornamental quince (Chaenomeles sp.) collected at Highmoor Farm, Maine.

Virginia Crab 9-23: Collected from a non-pitted tree at Highmoor Farm, Maine. It has indexed negative for chlorotic leaf spot, line pattern, rubbery wood and stem pitting.

Antonovka: Seedlings from open pollinated seed of an Antonovka tree at Highmoor Farm, Maine.

All apple virus isolates and indicators were maintained on seedling apple stocks at Highmoor Farm. Some of the isolates were top-worked to Antonovka seedling trees located in a virus-isolate-block-planting. Others were whip-grafted or budded to apple seedlings.

In indexing tests where apple indicator varieties

were used, a technique was followed similar to that suggested by Posnette and Cropley (1954). It involved the insertion of 2 apple buds into seedling apples. The top bud constituted the apple bud used as the indicator and a lower bud, put directly below, consisted of the variety to be indexed. The seedling was cut back to the indicator bud immediately after healing in of both buds in greenhouse tests, or the following spring in field tests, and readings were taken from the forced indicator shoot growth. In some cases the indicators were budded directly with the virus isolates. This was done by inserting the material to be indexed directly into 1-2 year old indicators propagated on seedling rootstocks. This method was used with indicators such as R-12740-7A as they are extremely sensitive to certain isolates, and bud development frequently fails. A more complete syndrome is possible when larger indicators are used, permitting the measurement of stem pitting, terminal dieback, and other growth responses that can be used as keys to differentiate virus isolates.

Where readings for stem pitting were made the inoculated indicator was sacrificed and the bark was completely removed from the point of bud union up to the first side shoot or at least to a minimum of 1 foot where possible. In taking readings for pitting on the sensitive R-12740-7A clone, this was not always possible due to extreme stunting and lack of shoot development.

Growth measurements were taken with a micrometer.

Stem diameters were sampled 1 inch above the bud union late in the growing season and recorded directly from the calipers in diameter-inches.

Heat therapy of infected budwood was carried out using both hot water treatments and hot air exposures. In attempts to inactivate stem pitting with hot water, 20 uniform terminal bud sticks having 20 buds per stick were cut from a stem-pitted Virginia Crab tree in late August. These were randomized into 4 bundles and the bud sticks were then cut into 4 portions with 5 buds per section and were labeled A, B, C, and D. The separate sections were then immersed into a hot water bath maintained at 50° C. for periods of 5, 10 and 20 minutes, again in a randomized order of treatment. One set was used as controls and received no heat treatment. The treated bud stick sections were successively removed from the water bath and 2 buds from each bud stick section were immediately budded to apple seedlings directly below non-pitted Virginia Crab indicator buds. The seedlings were cut back to the indicator bud the following spring, and readings for stem pitting transmission were made from shoot growth of the indicators.

Hot air therapy was accomplished by using a controlled temperature chamber built by the author, similar to one described by Fridlund (1962). The temperature was maintained at 100° F., plus or minus 1 degree. Young potted seedling apple trees which had broken dormancy were single budded with Northern Spy or Baldwin, both known to contain

chlorotic leaf spot but free from stem pitting factors. Eighteen budded seedlings of each variety were exposed for 26 days to 99-101° F. temperatures. They were then removed and the seedling apples were cut back to the inserted buds. The trees were kept under greenhouse conditions and buds from shoots were then indexed at Durham, New Hampshire, for chlorotic leaf spot on M. platycarpa and R-12740-7A indicators.

Radiation treatments were also attempted to inactivate stem pitting and dapple apple in infected dormant apple scions. Forty scions each from stem pitted Virginia Crab and dapple apple infected R-12740-7A dormant trees were cut in January 1962, randomized into 4 bundles of 10 scions each, and sent to Dr. D. G. Lundgren, Department of Bacteriology and Botany, Syracuse University, Syracuse, New York. Three bundles of each variety received separate radiation treatments from a cobalt 60 source, varying from 25,000 to 50,000r. These treatments were suggested based on previous studies with corn meristems by G. F. Cooper, Department of Botany and Plant Pathology, University of Maine, Orono, Maine. A fourth bundle of untreated bud sticks served as a control. The radiation-treated scions were then cut into 3 parts and whip-grafted to dormant apple seedlings 8 days after they were originally cut from the infected trees. The 240 whip-grafted scions were stored in moist peat moss for 2 weeks at 60° F. and then "lined out" in a greenhouse bench to break dormancy.

Since no known reports existed in the literature in regard to the production of antisera using apple tissues as antigen sources, an attempt was made to obtain antisera for use in determining serological relationships of apple virus isolates.[1] In order to have a known, healthy, virus-tested clone to serve as a control in serological tests for the stem pitting studies, the author was limited to a MM 104 rootstock/Cortland mosaic combination.

Rooted shoots of a MM 104 clone, obtained from the New York Agricultural Experiment Station and indexed as free from all known apple viruses, were planted at Highmoor Farm in the spring of 1962. They were inoculated in August with a bud from the Cortland mosaic isolate containing stem pitting, mosaic, and chlorotic leaf spot factors. The budded rootstock plants were harvested after leaf fall and stored at 3° C. until December 1962 at which time they were brought to Durham, New Hampshire, and grown in water cultures in aerated 1-gallon containers using Hoagland's No. 1 solution as a nutrient source (Fig. 2).

Root tissues were used in antigen preparations as it was thought they might contain less tannins, that possibly virus titre might be higher due to lower environmental temperatures, and that roots would offer uniform samples of tissue and could be obtained without loss in vigor to the plants sampled. Fulton (1941) found that roots

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[1] Petal preparation of an apple antiserum has been reported by Tremaine *et al.* (1963).

of systemically infected plants also contained virus. McCrum (1962b) found that mosaic and stem pitting viruses would transmit through root tissues. Two grams of young root tips were cut from 4 of the Cortland mosaic inoculated MM 104 plants. The tissue was immediately ground in liquid nitrogen, as Sanger (1962) reported this method to be superior with some unstable plant viruses. The ground, frozen powder was added to 10 ml. of a buffered solution containing .04M $K_2HPO_4 \cdot 3H_2O$, .01M sodium diethyldithiocarbamate and .01M thioglycolic acid (Fulton, 1959; Kegler and Opel, 1962) and homogenized in a Servall Omnimixer for 30 seconds at approximately 15,000 rpm. The homogenized root solution was then centrifuged for 10 minutes at 5,500 rpm. in a refrigerated, Model RC-2, Servall centrifuge. The supernatant was then filtered through glass wool and centrifuged at approximately 50,000 rpm. in a Spinco Model L centrifuge for 90 minutes. The supernatant was then poured off and the remaining pellet was resuspended in 2 ml. of the original buffer solution with the aid of a ground glass tissue homogenizer.

The suspended pellet solution was separated into 2 lots, 1 for intravenous injection and 1 for subcutaneous injection. One rabbit was injected intravenously with 0.5 ml. of the resuspended pellet in a marginal ear vein with a No. 26 needle. The remainder of the resuspended pellet, 1.5 ml., was mixed with 1.7 ml. of a Drakeol 6-VR:Arlacel A (9:1) (McKinney and Davenport, 1961) adjuvent and homogenized on a Brown Emulsor (Andovian Associates, Waltham, Mass.)

for 45 minutes and injected subcutaneously with a No. 20 needle into 4 separate locations in the abdominal region of a second rabbit. Both rabbits received 2 additional, similar preparations at 2-week intervals following the original injections. The rabbits were bled 10 days following the last injection by cardiac punctures using a No. 18 needle. The extracted blood was put into sterile test tubes and stored overnight in a refrigerator. Serum was obtained the following day from the clotted blood, put into sterile screw-top test tubes, frozen in dry ice and alcohol, and stored at dry ice temperature. Approximately 16 ml. of serum was obtained from 35 ml. of blood. During the course of bleeding, the subcutaneously injected rabbit died, thus making it necessary to terminate this portion of the study.

Preparation of the dapple apple antiserum was carried out in a similar manner, with the exception of the type of plant tissue used. Instead of root tissues, 5 g. of leaves from a R-12740-7A tree showing chlorotic leaf spot symptoms, which had previously been inoculated with the dapple apple isolate, were used for the immunizing antigen.

Test antigens were prepared according to an acetone-nicotine extraction method reported by Cadman (1959) which was found to be successful in serological studies where the extraction of virus was complicated due to the presence of plant tannins. Recently, Cropley (1963) demonstrated sap transmission of virus from apple tissues to herbaceous hosts when nicotine was used as a sap diluent.

Test antigens were made using both leaf and root

tissues. Apple tissues, cut in small pieces, were added to a 2.5% nicotine solution. A ratio of 1 g. of plant tissue was added to 4 ml. of the nicotine solution and homogenized in a Servall Omnimixer for 30 seconds at a speed of 15,000 rpm. The macerated tissues, plus nicotine, were squeezed through muslin and the extracted solution was then centrifuged for 5 minutes at a rheostat setting of 60 in a Servall SS-1 centrifuge. The precipitate was discarded and the clarified supernatant was combined with an equal part of acetone. The acetone-nicotine plant extract was then centrifuged for 10 minutes at 5000 rpm. in a Servall, Model RC-2, refrigerated centrifuge. The supernatant was discarded and the acetone precipitate was resuspended in distilled water in an amount equivalent to 1 part of original plant tissue to 2 parts of distilled water. The resuspended precipitate was then dialyzed overnight in running tap water at a temperature of 70° F. Dialyzing tubing used was Will, catalog No. 10886, size 28. After dialyzing, antigens were either frozen in sealed ampules in an alcohol-dry ice bath and stored at dry ice temperature (Hollings and Lelliott, 1961), or stored for short periods under refrigeration. The MM 104 mosaic root antigen was not subjected to acetone precipitation but was dialyzed in 2 changes of 1000 ml. of distilled water and concentrated by freeze drying.

The complement fixation technique described by Mathews (1957), and Boyd (1956) was selected for serological assay trials. Several factors influenced the selection of

this method: the complement fixation reaction is generally considered more sensitive than the precipitin reaction (Bawden, 1950 and 1964); preparations could not be checked for infective virus, and it was expected that the amount of virus contained in the preparations would be extremely low in titre.

Guinea pig sera used as a source of complement, and the hemolysin preparations were kindly supplied by Dr. T. G. Metcalf, Department of Microbiology, University of New Hampshire. Fresh sheep red blood cells were obtained from animals belonging to the University of New Hampshire, and preserved in Alsever's solution. The red blood cells were washed and centrifuged in 2 changes of 0.85% NaCl after initial centrifuging.

Dilutions of antisera and antigen were made up in tubes using saline. Tubes containing the antisera-antigen-complement mixtures were incubated for 1 hour at 37° C. in a water bath, or overnight at a temperature of 4° C., before adding the indicator system containing the sheep red blood cells and standardized hemolysin. The tubes were then incubated for 30 minutes at 37° C. Then the tubes were removed from the water bath and relative readings of the amounts of complement fixed by the antigen-antisera reaction were made. Readings were based on the amount of hemolysis that occurred and were graded visually. A reaction rated as 4 (++++) was equivalent to the sheep red blood cell control, 2 (++) indicated 50% of red blood cells lysed, and a rating

of 0 indicated complete sparkling hemolysis had taken place.

All tests included the following controls: complement at 2, 1, and 0.5 full units; hemolysin, sheep red blood cell, and serum and antigen anticomplementary controls. Additional controls were run using normal rabbit sera as well as antigens from healthy homologous roots and leaves. All test sera were incubated at 56° C. for 1/2 hour before use to inactivate normally occurring complement. The amounts of reagents used in running the complement fixation tube tests can be found in the Appendix.

# SECTION IV

## EXPERIMENTAL RESULTS

Names ascribed to disease symptoms reported in the results are those commonly used by workers in the field of apple virus research. Photographs of symptoms and descriptions of the various disease syndromes referred to can be found in Maine Agricultural Experiment Station Bulletin 595, New Hampshire Agricultural Experiment Station Technical Bulletin 101, and in Technical Communication No. 30, Commonwealth Bureau of Horticulture and Plantation Crops, East Malling, England.

In general, virus disease of apple can be grouped into 3 categories based on the type of tissue affected:- diseases having characteristic symptoms of (1) leaves, (2) fruits, and (3) stems. In determining the causal relationships of fruit diseases to those of leaf and stem disorders, it is necessary to have fruit production on indicators. Inoculations to fruit indicators have been made during the course of this study but to date there has been no developing fruit, consequently disease reactions reported with virus isolates fall into the leaf and stem symptom categories.

### 1. Presence of Latent Virus in Indicators

One of the first problems to arise in the attempt to classify the causal relationship of stem pitting to other apple diseases was to obtain indicators which did not contain latent viruses. Some of the indicators which were collected for trials were later found to be carrying one or more latent viruses.

Since the results from the cross-indexing trials (Table 1) indicated that future host index tests could be run with only a limited number of indicators, a further search for additional indicators was attempted using seedling sources.

Table 1. Presence of Virus Infection Revealed when Selected Indicators were Cross-indexed on 3 Apple Indicators.

| Indicator Clone | Symptoms occurring on | | |
|---|---|---|---|
| | R-12740-7A (Purdue) | Virginia Crab 9-23 (Maine) | *Malus platycarpa* (Long Ashton) (Geneva) |
| *M. platycarpa* (Long Ashton) | 0[a] | 0 | -- |
| *M. sieboldi* No. 13 seedling | 0 | 0 | 0 |
| Lord Lambourne (Geneva) | 0 | 0 | -- |
| Lord Lambourne PI 203815 | -- | SP | -- |
| Lord Lambourne PI 205048 | -- | SP | -- |
| Lord Lambourne PI 238789 | -- | 0 | LP |
| Virginia Crab 18-2 (Maine) | SP, CLS | 0 | LP |
| Virginia Crab C-9 (Purdue) | 0 | 0 | 0 |
| Virginia Crab 9-23 (Maine) | 0 | -- | 0 |
| Cox's Orange PI 238787 | CLS, SP | 0 | 0 |
| Belle de Boskoop PI 199687 | CLS, SP | SP | LP |

[a] The meaning of the various symbols are: SP-stem pitting, CLS-chlorotic leaf spot, LP-line pattern, 0-no symptoms observed, Dash-no test made.

## 2. Seedlings of *Malus* sp. as Stem Pitting and Mosaic Indicators

Seed transmission of apple viruses has not been reported; therefore, it was thought that if a stem pitting-sensitive clone derived from a seedling could be found it would in itself be free of latent virus and thus remove the potential danger of virus interference.

Seedlings of 2 crab apple species and from an open pollinated Robin Crab were tested as possible indicators for stem pitting. The seedlings were not budded directly as this would preclude the future use of any seedling clone found to be a good indicator. Instead, 2 buds from each seedling were budded to commercial apple seedlings and inoculated by the double budding method. Results from using the 3 seedling crab sources are shown in Table 2. Only 1 seedling indicator clone was found to be useful as an indicator for stem pitting. This clone, designated as *M. sieboldi* No. 13, exhibited severe pitting as well as dwarfing. In obtaining readings for stem pitting some shoot growth of the indicator is necessary; an indicator that reacts to virus inoculum to such slight degree that little or no stem growth is produced would, of course, not be satisfactory. Since the *M. sieboldi* No. 13 seedling produced readable shoots, it has been saved and increased for future tests as a stem pitting indicator.

Table 2. Reaction of Seedlings from 2 *Malus* sp. and Seedlings from Robin Crab when Inoculated with a Stem Pitting Virus Isolate.

| Seedling Variety | No. Seedlings Tested | No. Seedlings Pitted |
|---|---|---|
| *M. sieboldi* arborescens | 14 | 1 |
| *M. floribunda* | 24 | 0 |
| Robin Crab | 14 | 14[a] |

[a] All 14 Robin Crab seedlings exhibited a very fine "pin point" type of pitting. It was not known whether this was due to the virus, as similar pitting was observed on some clonal seedlings from which the indicator buds were taken.

While conducting tests with apple mosaic indicators it was often noted that only the first developing leaves on indicator shoots displayed mosaic symptoms. Leaves formed later in the growing season were symptomless. In some cases only 1 or 2 spots of mosaic would occur on a single leaf of an indicator. Frequently, however, shoots that arose from the double-budded seedling rootstock used in indexing would exhibit strong mosaic patterns on many leaves. As a consequence a search was made to find a suitable mosaic indicator that would produce symptoms on leaves throughout the growing season. This was thought to be an important criterion because in warmer apple growing areas of this country symptom expression of apple mosaic is completely suppressed.

Seedlings from fruit of an Antonovka tree at Highmoor Farm had shown particularly good symptoms when used as rootstocks in indexing tests. Because several of these seedlings, which had never been budded, were available, 43 of them were clonally checked for use as mosaic indicators. Two buds from each seedling were budded to commercial apple seedlings and inoculated with mosaic. Of the 43 seedling clones checked, 5 were found to show good mosaic symptoms on successive developing foliage, 3 exhibited fair symptom development throughout the season, and the remaining 35 seedling clones varied as to intensity and number of leaves affected. When the same clones were rechecked in the field the following year, only 2 clones were found to produce good mosaic symptoms on leaves throughout the season. These 2 clones, designated Antonovka #1 and Antonovka #24, were saved and planted in an indicator block for comparison studies with other apple mosaic indicators.

### 3. Graft Transmission Studies with Stem Pitting

A. Location of Virus in Buds. Pierce's disease of the grapevine (alfalfa dwarf disease) is reported to be restricted to woody tissues (Esau, 1948). Stem pitting of apples is also characterized by abnormal stem tissues resulting in wood deformation. In order to determine whether woody tissues were needed in transmitting virus in bud inoculations, the following experiment was conducted.

Fifteen apple seedlings were double budded in the

field with non-pitted Virginia Crab buds, as well as with inoculum buds cut from a stem pitting isolate containing a wood chip. A second set of 15 seedlings received buds of non-pitted Virginia Crab and were double budded with inoculum consisting of only the bud shield from which the wood chip had been removed. Data obtained from shoot growth on the indicators revealed (Table 3) that the stem pitting factor can be transmitted with either method and that the virus is located in bark and bud tissues. No tests were run to see if the virus could be transmitted with wood chips.

Table 3. Effect of Wood Chip in Inoculum as Compared to Bud Inoculum Minus Wood Chip in Transmitting Stem Pitting Virus to Virginia Crab.

| | Indicators | | |
|---|---|---|---|
| Type of Inoculum | No. Budded | No. Shoots | No. Pitted |
| Bud containing wood chip | 15 | 11 | 11 |
| Bud with wood chip removed | 15 | 12 | 11 |

When the stems were examined for stem pitting it was noticed that several of the seedlings in the set that contained the wood chip had developed a sort of "reverse wood pitting", consisting of numerous small outgrowths of woody tissue resembling pegs. This effect has been noticed in other transmission tests but no explanation is suggested.

B. Infection Period. The infection period for stem pitting (length of time that the inoculum bud must remain on the tree in order to demonstrate transmission) has not been determined, but data presented in Table 4 indicates that it requires a minimum period of at least 15 days. In this field experiment, 2-year old Virginia Crab, non-pitted trees were budded directly on the growing terminal shoot with a stem pitting isolate. The terminal shoot was then cut directly below the inoculation bud. This method was used, rather than simply rubbing off the bud, to remove the possibility of leaving any infected cells that might have remained to transmit the virus. Readings were taken by removing the entire bark at the end of the following growing season.

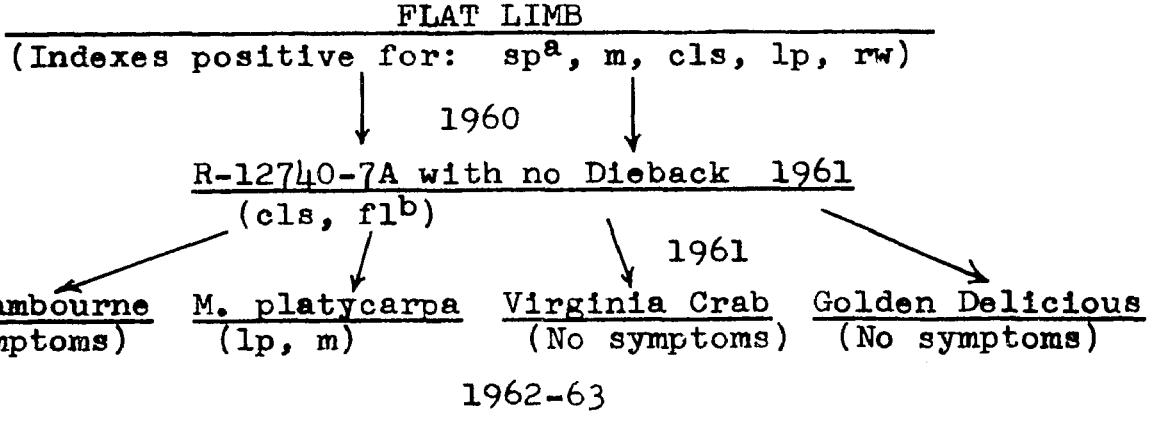
Table 4. Bud Inoculation and Removal as a Means of Determining the Infection Period Required for Stem Pitting Virus in 2-Year Old Virginia Crab Trees.[a]

| Time between Budding and Removal of Bud | Virginia Crab | |
|---|---|---|
| | No. of Trees Budded | No. of Trees Pitted |
| 1 hr. | 10 | 0 |
| 4 hrs. | 10 | 0 |
| 8 hrs. | 10 | 0 |
| 3 days | 10 | 0 |
| 7 days | 10 | 0 |
| 15 days | 10 | 0 |
| 1 year | 10 | 10 |

[a] Trees were budded in the field while actively growing.

In an earlier report (McCrum, 1962a) it was observed that a flat limb isolate caused dieback and chlorotic leaf spot when budded to the R-12740-7A indicator. This same virus isolate also contained stem pitting virus and in two cases where bud union of the flat limb isolate apparently failed, no dieback occurred in the indicators, although chlorotic leaf spot was evident. Further indexing of the R-12740-7A indicators where bud union of flat limb was not successful (Fig. 6) offers additional evidence to suggest that bud transmission of stem pitting requires a certain minimum infection period longer than that required for mosaic or chlorotic leaf spot. It also indicates that dieback of the other R-12740-7A indicators was due to the stem pitting factor since stem pitting could not be recovered from the R-12740-7A indicator that failed to display dieback symptoms.

Figure 6. Re-Indexing of an R-12740-7A Indicator Previously Inoculated with a Bud from a Flat Limb Isolate that did not Unite.




[a] Symbols signify the following: sp-stem pitting, m-mosaic, cls-chlorotic leaf spot, rw-rubbery wood, lp-line pattern, fl-flat limb.

[b] Symptoms suggestive of flat limb were observed in 2-year old wood.

C. Recovery of Stem Pitting from Inoculated Quince. In a search for additional stem pitting indicators, quince seedlings were used. Potted seedlings were budded in the greenhouse in May 1959 and set out in the nursery row in June 1959. Stem pitting could not be recovered from quince buds in 1959 but was recovered in 1961 (Table 5). Several inoculated quince seedlings developed ring spots and line patterns in leaves formed in the first flush of growth (Fig. 3). Quince inoculated with non-pitted Virginia Crab displayed no symptoms. The ring spots and line patterns observed on the inoculated quince (Fig. 3) may have been due to the presence of other latent viruses contained in the stem pitting inoculum as budwood from the inoculated quince gave positive reactions when indexed on M. platycarpa and R-12740-7A. Budwood from quince inoculated with the non-pitted Virginia Crab 9-23 gave negative readings on M. platycarpa and R-12740-7A.

Table 5. Reactions of 3 Indicators to Quince Seedlings Previously Inoculated with Virginia Crab Buds in 1958.

| Indicators[a] | Quince Inoculated with stem pitted Virginia Crab | | Quince Inoculated with non-pitted Virginia Crab 9-23 |
|---|---|---|---|
| | Year Indexed | | Year Indexed |
| | 1959 | 1961 | 1961 (only) |
| Virginia Crab | 0[b] | SP | 0 |
| *M. platycarpa* | -- | LP | 0 |
| R-12740-7A | -- | CLS | 0 |

[a] Five of each indicator listed were double budded with quince inoculum buds.

[b] Symbols represent the following: 0-no symptoms observed, SP-stem pitting, LP-line pattern, CLS-chlorotic leaf spot, Dash-no test made.

D. Relation of Stem Pitting to Fruit Symptoms and and Seed Transmission. Although wood pitting of sensitive stocks is considered to be the diagnostic symptom of apple stem pitting, fruit occurring on stem pitted infected trees are sometimes affected. When a stem pitted Virginia Crab tree is allowed to fruit, the apples produced are smaller than normal and exhibit longitudinal grooves extending from the calyx end to the stalk. Infected Virginia Crab fruits tend to remain on the tree after healthy fruits have dropped. Fruit samples taken at random from 2 small Virginia Crab trees, 1 inoculated with stem pitting and the other left as a healthy check, show that length of fruit stem is reduced

due to stem pitting (Table 6). Fruits from the infected tree, reported in Table 6, also had a fine, netted, cobweb pattern of russet covering the entire fruit surface which could be seen only on close observation. Total weight of 20 fruits on the stem pitted tree was 1 pound, 5½ ounces, while 20 fruits from the healthy tree weighed 1 pound, 10 ounces. Although water displacement of both was the same, density of the pitted fruits was lower than that of the healthy fruits.

Table 6. Changes in Length of Virginia Crab Fruit Stems Due to Stem Pitting Infection.

| | Fruits from Stem pitted tree | Fruits from Non-pitted tree |
|---|---|---|
| Stem length in cm. | 2.5 | 4.3 |

Fruits of Red Delicious top-worked to pitted Virginia Crab trees sometimes display deep sutures which are not due to seed abortion (Fig. 4). The ratio of sutured fruits to normal fruits varies from season to season.

In limited indexing trials, standard Golden Delicious trees that consistently produce russeted fruits were compared with Golden Delicious trees that consistently produced fruits with good fruit finish. Only 5 trees from each category were indexed; however, all trees that produced russeted fruits indexed positive for stem pitting, whereas trees

which produced russet-free fruits indexed negative for stem pitting.

During the course of this study, hundreds of commercial seedlings have been budded with the Virginia Crab indicator, and in no case has evidence of stem pitting been observed on such indicators.

Seeds were harvested from deformed fruits of Red Delicious and pitted Virginia Crab fruits. Although seedlings obtained from such seeds were limited in number -- 38 from the pitted Virginia Crab, and 20 from the Red Delicious -- they did not transmit stem pitting when budded with healthy non-pitted Virginia Crab. Many of the seedlings arising from the pitted Virginia Crab were extremely dwarfed and could not be budded. Others displayed foliage and shoot growth suggestive of virus symptoms. Since the seedlings were from open pollinated seed, and Virginia Crab is reported to be a triploid parent, variations of this sort are to be expected (Edgecomb, 1938).

### 4. Transmission Studies with Selective Apple Indicators

Plants propagated by vegetative means, such as apples, have greater opportunities to become infected with viruses than do seed propagated plants. Except for a few cases, it is almost impossible to trace the history of a given variety of apple from the orchard tree back to the original parent germ plasm. Each time a variety is combined

with another plant in the course of budding and grafting operations necessary to maintain the original selection, its chances of contracting virus infection increases. It then becomes a problem when a "new" virus disease is reported to determine which virus or viruses actually present in the diseased apple are responsible for the specific disease symptoms described.

A. Relation of Virus Isolates to Indicator Symptoms. When budwood from trees exhibiting different virus disease symptoms were indexed for virus content, several interesting facts were obtained (Table 7). Stem pitting was found to be present in many of the isolates. Dapple apple existed separate from stem pitting. This confirmed information obtained in previous reports (McCrum, 1962a; Barrat, 1958). Isolate 1-12A induced pitting of Virginia Crab but did not produce chlorotic leaf spot when inoculated directly into 1 and 2-year old trees of R-12740-7A. This isolate also behaved similarly in previous tests conducted by Cation (1962). Cortland J-5 isolate and chlorotic leaf spot isolate both produced chlorotic leaf spot in R-12740-7A but did not induce pitting in Virginia Crab. The latter isolate reacted similarly in tests conducted by Mink (1962). The isolates Cortland I-12, Cortland J-30, green mottle, and flat limb produced a ring spot symptom in leaves of M. platycarpa in addition to line pattern. The first 3 of these virus isolates came from trees exhibiting fruit disorders. Only one

Table 7. Behavior of Selected Virus Isolates on Virginia Crab Compared to their Behavior on Other Latent Virus Indicators.[a]

| Virus Isolate | Indicators | | | |
|---|---|---|---|---|
| | Virginia Crab | R-12740-7A | Malus platycarpa | Lord Lambourne (N.Y.) |
| Green mottle | SP[b] | CLS | LP, RS | 0 |
| Russet ring | SP, VLF | CLS | LP | 0 |
| Rubbery wood | SP | CLS | LP | 0 |
| Granny Smith | SP, M | CLS | LP, M | -- |
| Erb mosaic | SP, M | -- | M | -- |
| Dapple apple | 0 | CLS | 0 | -- |
| Flat limb | SP, M | CLS, D | LP, RS, M | M, RW |
| Stem pitting | SP | CLS, D | LP | 0 |
| Chlorotic leaf spot | 0 | CLS | LP | -- |
| Cortland mosaic | SP, M | CLS, D | -- | -- |
| Scar skin (Maine) | 0 | 0 | 0 | -- |
| Quince 8-40 | SP | CLS | -- | -- |
| Quince mosaic | -- | M | M, LP | M |
| Cortland J-5 | 0 | CLS | 0 | 0 |
| Cortland I-12 | SP | CLS, D | LP, RS | 0 |
| Cortland J-30 | SP | CLS, D | LP, RS | 0 |
| Cortland E-27 | 0 | 0 | 0 | -- |
| 1-12 A | SP | 0 | -- | 0 |
| Dwarf fruit | SP | CLS, D | -- | 0 |
| LM-33 | SP | CLS, D | -- | 0 |

[a] Bud union or shoot growth of the inoculated virus isolate occurred in all cases where negative symptom readings were recorded; also a minimum of 5 indicators were tested with each virus isolate reported.

[b] The meaning of the various symbols are: SP-stem pitting, CLS-chlorotic leaf spot, VLF-vein leaf fleck, RS-ring spot, LP-line pattern, RW-rubbery wood, M-mosaic, D-terminal dieback which was obtained only when 1-2 year old trees were budded directly, 0-no symptoms observed on 1-year old indicators, and Dash-signifies no test made.

isolate, flat limb, indexed positive for rubbery wood; however, this symptom was observed in a 3-year old tree of Lord Lambourne. Other isolates tested on Lord Lambourne, including the rubbery wood isolate from New York, have had an incubation period of only 2 years and further readings taken on 3 or 4-year old inoculated trees may prove that rubbery wood is present in the other isolates. Rubbery wood has been shown to have a long incubation period (Mulder et al., 1955).

Data in Table 7 also indicates that a single isolate can carry several factors as shown by the production of 7 different reaction symptoms obtained with the flat limb isolate. Isolates that indexed positive for stem pitting and chlorotic leaf spot also induced dieback of R-12740-7A shoots when 1 and 2-year old trees were inoculated directly (Fig. 5). Little or no growth occurred on the latter indicators when they were used in double budding index tests. Although R-12740-7A is very sensitive to many virus isolates, an isolate containing quince mosaic (Fig. 8) did not appear to affect the growth of this indicator when 1 and 2-year old trees were inoculated directly. Russet ring produced an additional symptom on Virginia Crab which was designated "vein leaf fleck", (Fig. 7). It was somewhat similar to chlorotic leaf spot symptoms observed in R-12740-7A.

The isolate called "scar skin" (Maine) was obtained from a Red Delicious tree exhibiting fruit symptoms (Figs. 9

and 10) which appeared identical to those illustrated by Millikan (1963). It has been proven to be graft transmissible, having caused fruit symptoms similar to scar skin to appear on healthy trees the year following inoculation with infected scions. This particular isolate indexed negative on all indicators tested as reported in Table 7.

Figure 2. MM 104 apples grown in aerated one gallon containers using Hoagland's No. 1 solution as a nutrient source.

Figure 3. Stem pitting in seedling quince, showing ring spot and line pattern.

Figure 4. Pitting on fruit of Red Delicious top-worked to pitted Virginia Crab.

Figure 5. Terminal dieback in 1 and 2-year old trees of R-12740-7A inoculated with stem pitting. Healthy control trees on left.

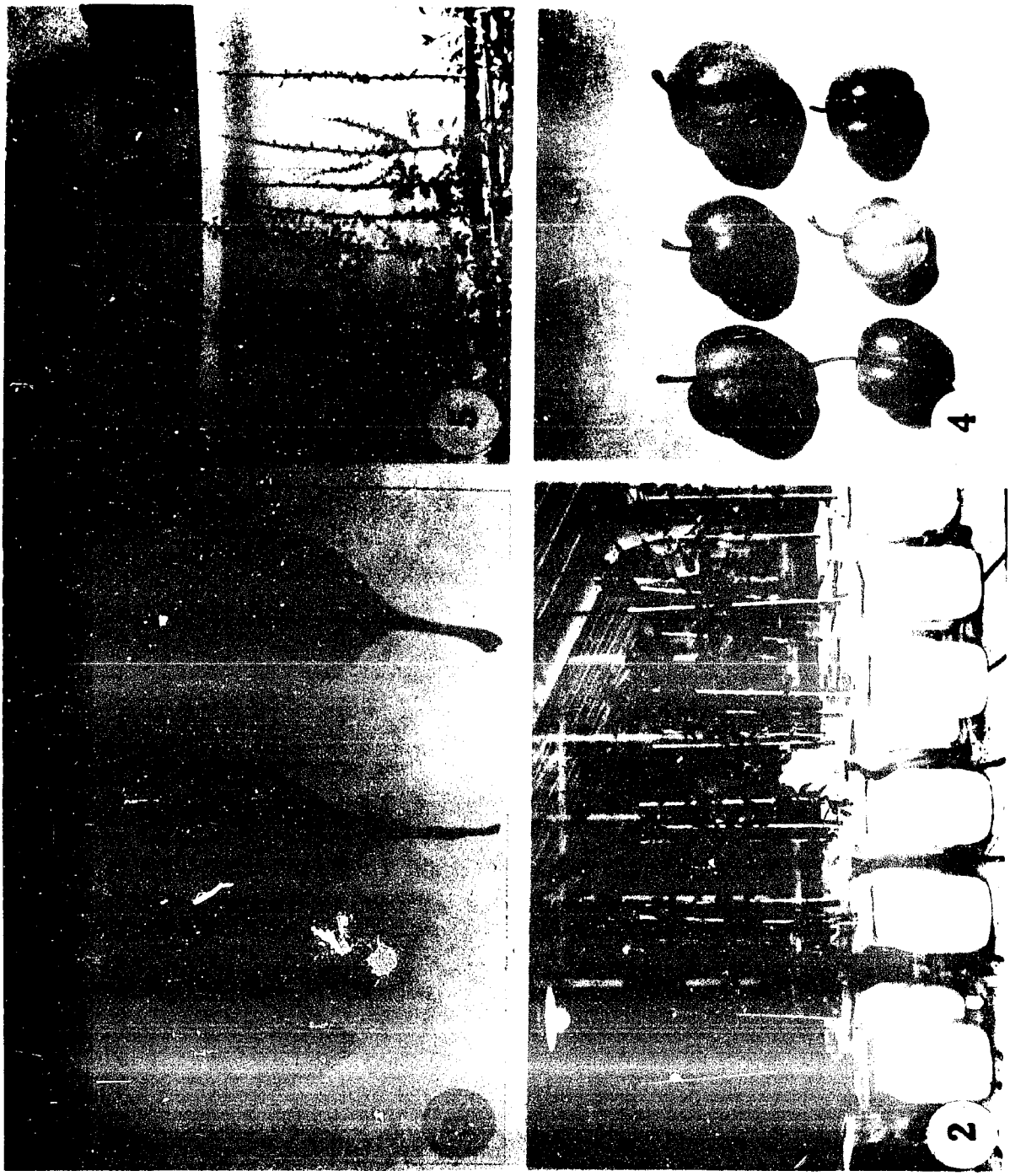

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Figure 7. Russet ring in Virginia Crab; three leaves on left with "vein leaf fleck", healthy leaf on right.

Figure 8. Quince mosaic in seedling apple left and right, naturally infected quince in center.

Figure 9. Scar skin in Red Delicious.

Figure 10. Scar skin in Red Delicious, calyx end.

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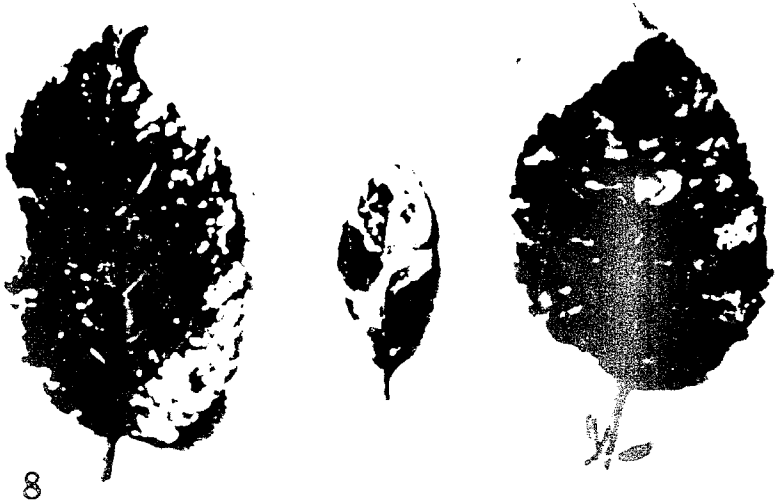

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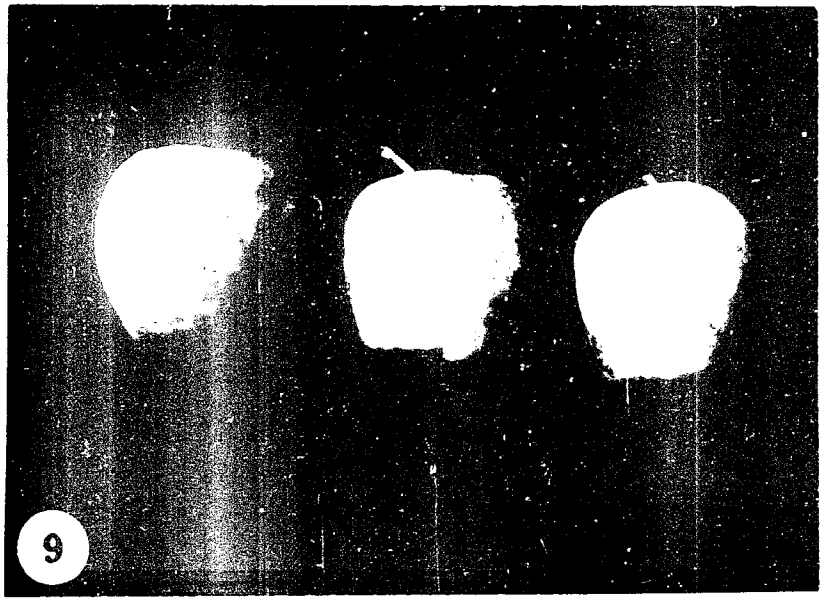

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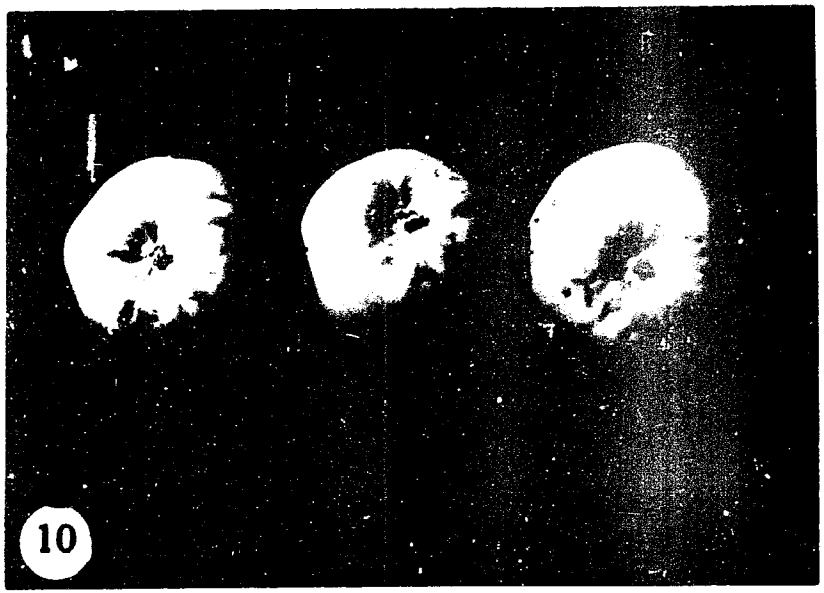

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B. Growth Reaction of Indicators Inoculated with Apple Virus Isolates. Several isolates were indexed on Virginia Crab, M. platycarpa and R-12740-7A in field tests in 1961, using the double budding method, to determine the effect of virus on indicator growth. Measurements taken over a 2-year period (1962-63) are reported in Table 8. The R-12740-7A indicator was extremely sensitive to isolates tested. Only a small amount of shoot growth occurred in the first year and no increase in growth was recorded in the second year. The extreme stunting and growth of this indicator, as well as that for the other 2 indicators, is graphically depicted in Figs. 11, 12, 13 and 14. Although only slight differences occurred in the first year's growth of the Virginia Crab and M. platycarpa indicators, the effects of virus infection on growth were greatly magnified in the 2-year old indicators.

Isolates Granny Smith and Green mottle were highly infectious in Virginia Crab. No increase in growth was recorded for the 2-year old indicators, and death of several indicators occurred. Russet ring infected indicators made little growth in the second year. Dapple apple, which did not induce pitting in Virginia Crab, did cause a reduction in growth of Virginia Crab, as did the Erb mosaic and rubbery wood isolates. Dapple apple did not cause a reduction in the growth of M. platycarpa over a 2-year period. This indicator has reacted similarly to the same virus isolate in other trials.

Table 8. Growth of 3 Apple Virus Indicators Inoculated with Apple Virus Isolates Over a 2-Year Period and Measured in Diameter-Inches.

| Isolate | Indicator Growth | | | | | |
|---|---|---|---|---|---|---|
| | *M. platycarpa* (Long Ashton) | | Virginia Crab 18-2 | | R-12740-7A | |
| | 1st yr. | 2nd yr. | 1st yr. | 2nd yr. | 1st yr. | 2nd yr. |
| Controls[a] | 0.53[b] | 0.90 | 0.50 | 0.97 | 0.50 | 0.91 |
| Dapple apple | 0.50 | 0.90 | 0.49 | 0.84 | --[c] | -- |
| Rubbery wood | 0.35 | 0.58 | 0.42 | 0.79 | 0.15 | D[d] |
| Erb mosaic | 0.43 | 0.64 | 0.42 | 0.84 | -- | -- |
| Russet ring | 0.37 | 0.49 | 0.37 | 0.43 | 0.14 | D |
| Green mottle | 0.35 | 0.40 | 0.36 | D | 0.18 | D |
| Flat limb | 0.32 | 0.41 | -- | -- | -- | -- |
| Granny Smith | 0.37 | 0.51 | 0.39 | D | 0.15 | D |

[a] Controls consisted of uninoculated indicators; other isolates refer to those cited in Materials and Methods.

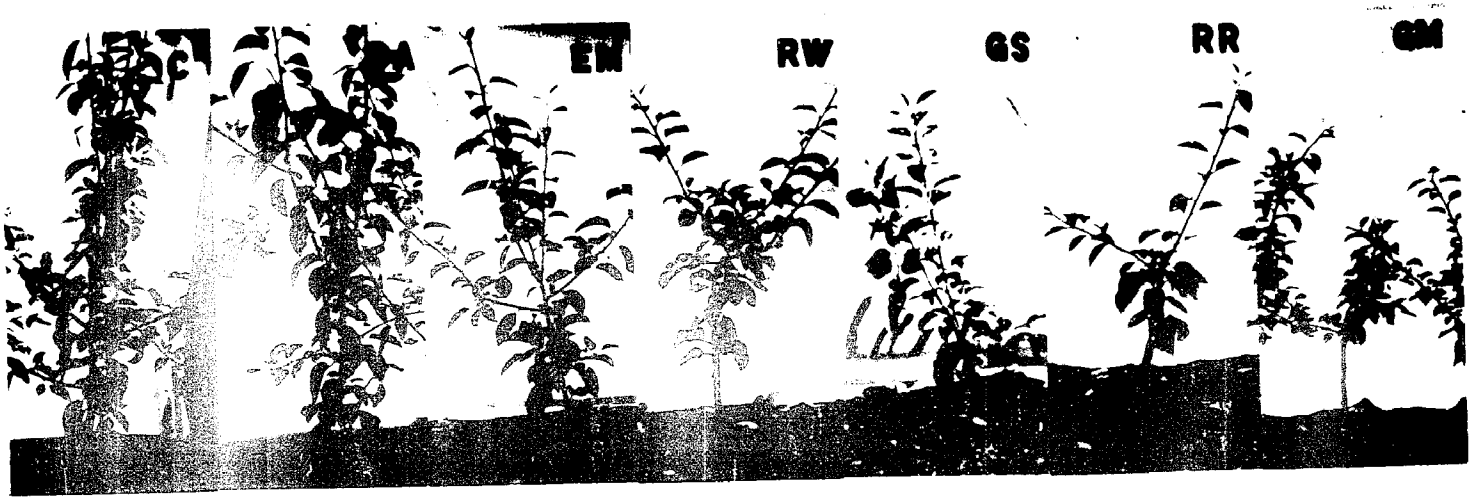
[b] Refers to the average diameter of 5 indicators.

[c] No tests run on these indicators, as signified by a dash.

[d] D-indicators were dead, or no increase in growth occurred at the end of the 2nd year.

Figure 11. Effect of various apple virus isolates on the growth of Virginia Crab: C-control, DA-dapple apple, RW-rubbery wood, EM-erb mosaic, GS-Granny Smith, RR-russet ring, GM-green mottle.

Figure 12. Effect of various apple virus isolates on the growth of Malus platycarpa (Long Ashton): C-control, DA-dapple apple, EM-Erb mosaic, RW-rubbery wood, GS-Granny Smith, RR-russet ring, FL-flat limb, GM-green mottle.

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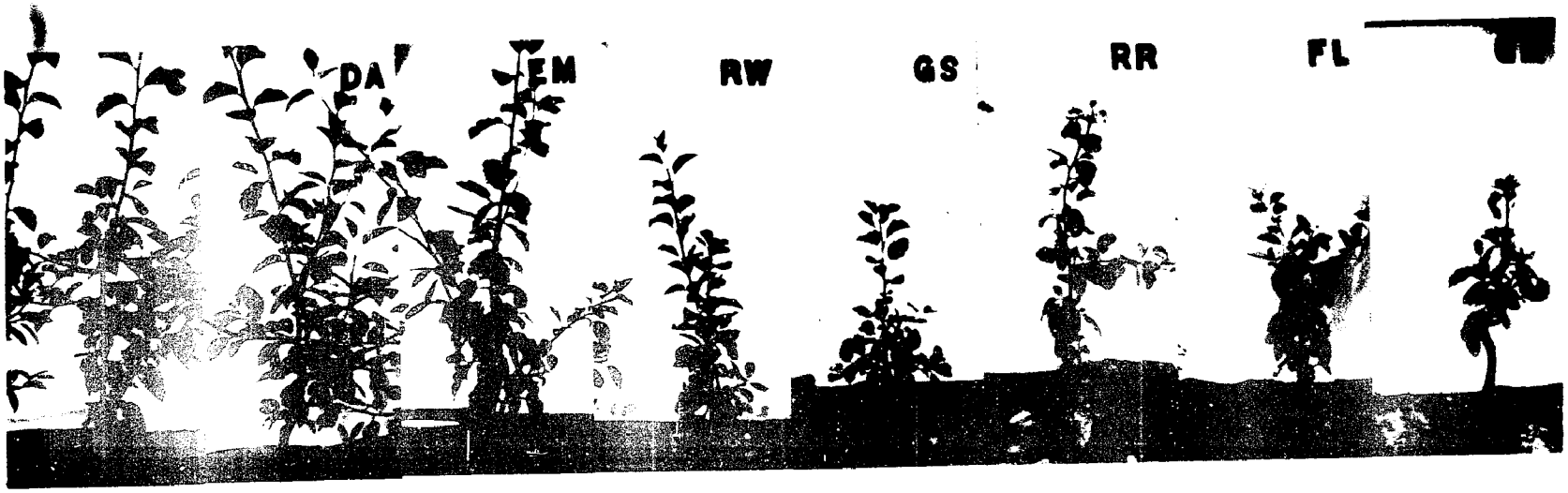


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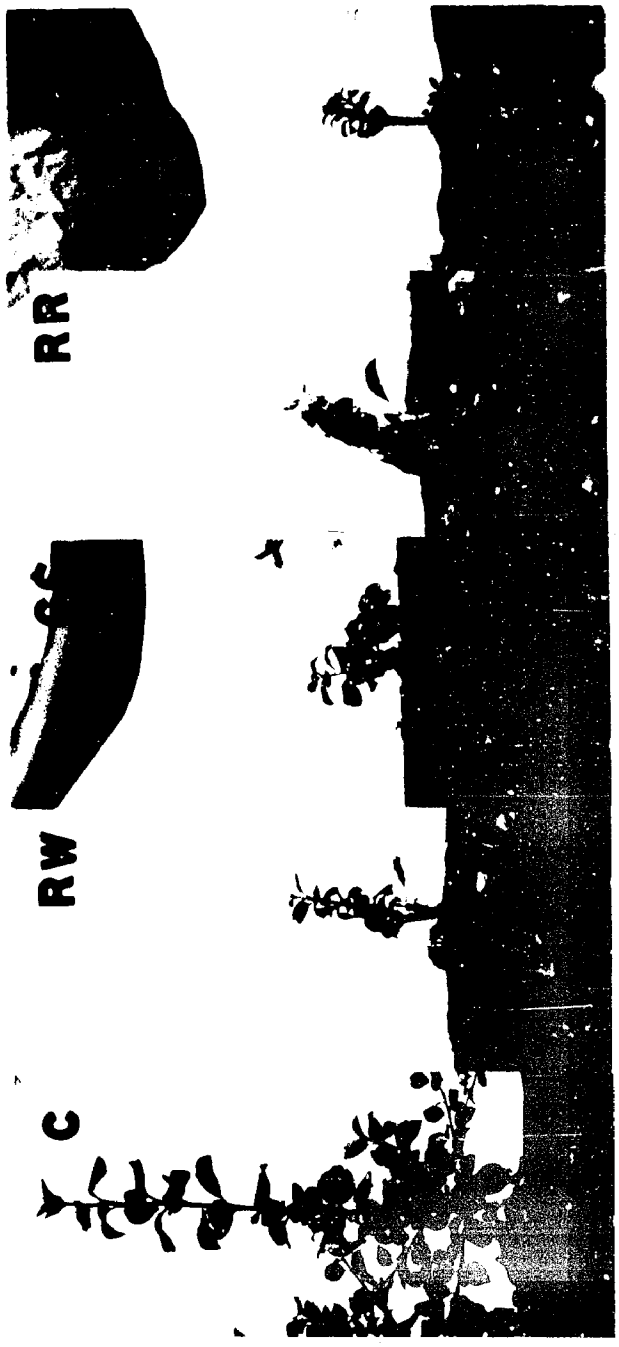
Figure 13. Effect of various apple virus isolates on the growth of R-12740-7A: C-control, RW-rubbery wood, GS-Granny Smith, RR-russet ring, GM-green mottle.

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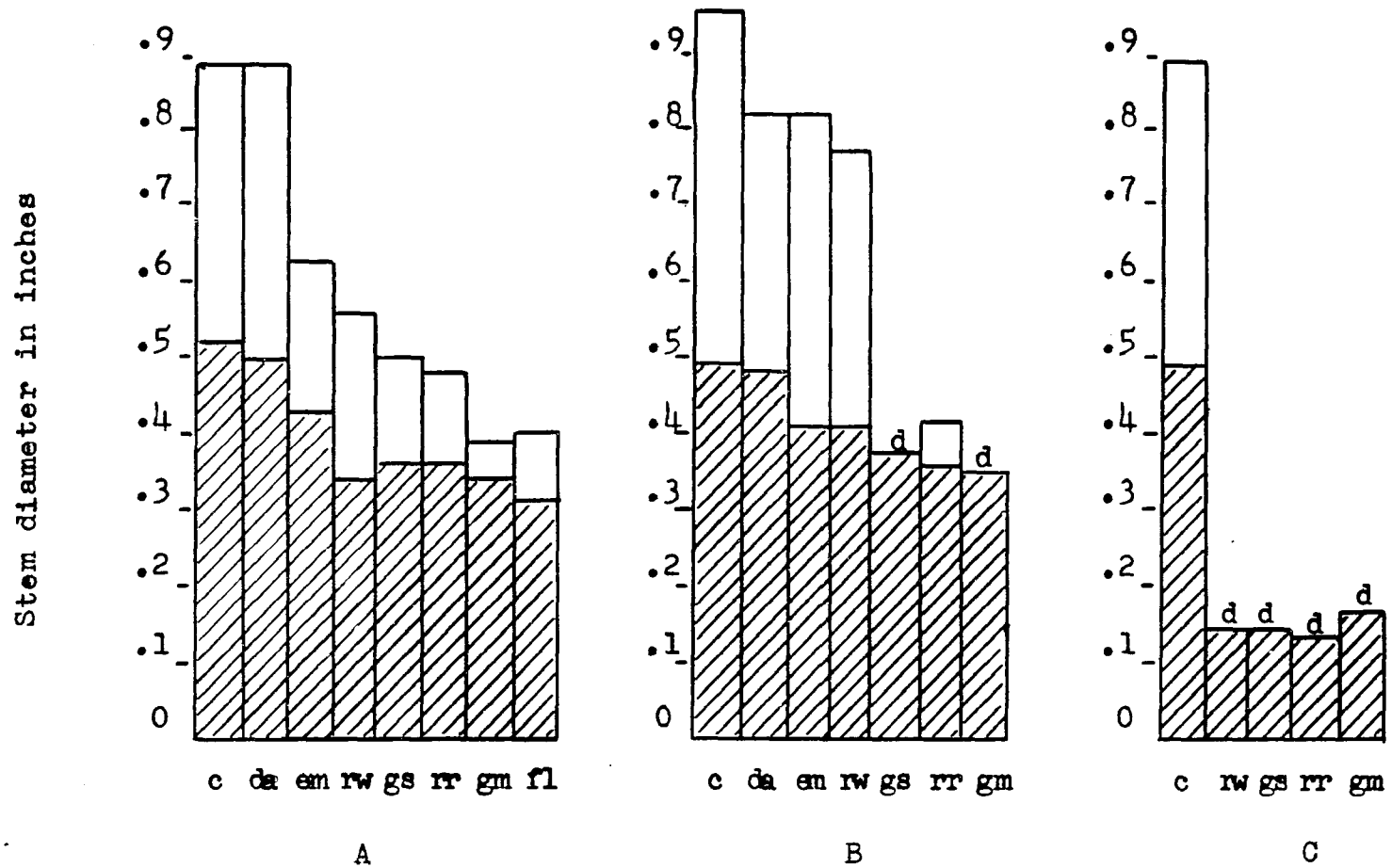


Fig. 14. Growth of A) Malus platycarpa (Long Ashton), B) Virginia Crab 18-2, and C) R-12740-7A indicators, measured in diameter-inches of shoot growth, over a 2-year period, inoculated with apple virus isolates containing: da, dapple apple; em, Erb mosaic; rw, rubbery wood; gs, Granny Smith; rr, russet ring; gm, green mottle; and fl, flat limb. Crossed portion of bar -- 1st year's growth. Plain portion of bar -- 2nd year's growth. Uninoculated indicator growth is represented by the symbol c, and d indicates no growth occurred in the 2nd year.

## 5. Serology

All complement fixation tests were conducted with serum obtained from rabbits injected subcutaneously.

A. Cortland Mosaic-Stem Pitting Antisera Tests. Preliminary tests in which complement and the homologous MM 104 root antigen controls were run using undiluted antigen resulted in anticomplementary action. Dilutions of the antigen extract used at 1:2 (1 part antigen to 1 part saline) did not result in anticomplementary activity. The homologous MM 104 root antigen was prepared by dialyzing the clarified-nicotine-extracted sap against 2 changes of 1000 ml. of distilled water and concentrating the sap by freeze drying. Antigens that were prepared by acetone precipitation followed by dialyzing the resuspended precipitate in tap water did not show anticomplementary activity when used undiluted.

Complement fixing activity of the homologous MM 104 root extract test antigen was obtained at dilutions of 1:30 when tested with the anti-mosaic-stem pitting sera used at a 1:2 dilution, although results equivalent to the sheep red blood cell control (++++) were obtained only at a dilution of 1:5, Table 9. A stronger positive reaction resulted (Table 10) when the homologous MM 104 root extract test antigen was incubated overnight with the antiserum and complement at 4° C. before adding the indicator system (hemolysin and sheep red blood cells) than when the indicator

Table 9. Complement Fixation Activity of Anti-Mosaic Stem Pitting Rabbit Serum with the Homologous Root Extract Test Antigen.[a]

| Root extract dilutions | Serum | |
|---|---|---|
| | Anti-mosaic-stem pitting (1:2) | Normal rabbit[b] (1:2) |
| 1:5 | 4[c] | 0 |
| 1:15 | 2 | -- |
| 1:30 | T | -- |
| 1:45 | 0 | -- |

[a] The antigen-antibody-complement reagents were incubated overnight at 4° C. before adding the indicator system.

[b] No anticomplementary action occurred in the antigen and antiserum controls when used at dilutions of 1:5. No hemolysis took place in the sheep red blood cell or hemolysin controls.

[c] Rating: 4-equal to the sheep red blood cell control (++++), 2-one half of the red blood cells not hemolyzed (++), T-trace of red blood cells remaining, 0-sparkling hemolysis, and a dash signifies that no test was made.

system was added after incubation for 60 minutes at 37° C. Additional preparations with the homologous antigen source were not possible as new roots had not developed and plants had started to decline. Healthy MM 104 rootstocks maintained under the same conditions, however, continued to produce new root growth.

Since the original immunizing antigen source had been inoculated with an isolate containing stem pitting and mosaic factors, the antiserum was checked for activity with test antigens known to contain these factors. Two test antigens, 1 from a seedling apple inoculated with Cortland mosaic and 1 from the 1-3B isolate (containing stem pitting but not mosaic) gave positive complement fixing reactions with the mosaic-stem pitting antisera. No reaction occurred when a healthy MM 104 leaf test antigen was used, nor did any of the test antigens react with normal serum in fixing complement (Table 10). Slight activity of the healthy MM 104 root test antigen was noted at dilutions of 1:2 and 1:4 (Tables 10 and 11).

The MM 104 mosaic-stem pitting infected root test antigen, prepared by the distilled water dialyzing-freeze dry concentration procedure and stored in screw-top vials at a temperature of 4° C., lost activity after 14 days (Table 11). However, the Cortland mosaic seedling antigen and the 1-3B antigen prepared by the acetone precipitation-tap water dialysis method and stored under similar conditions remained active for at least 27 days (Table 12).

Table 10. Comparison of Healthy Root and Leaf Antigens vs. Antigens Containing Mosaic and Stem Pitting with Mosaic-Stem Pitting Antiserum and Normal Rabbit Serum.

| Test Antigens (1:5) | Serum Dilutions | | | | | Anticomplementary activity of antigens (1.5)[a] |
|---|---|---|---|---|---|---|
| | Mosaic-Stem Pitting | | | Normal Rabbit | | |
| | 1:2 | 1:4 | 1:8 | 1:2 | 1:4 | |
| MM 104 leaves (healthy) | 0[b] | 0 | 0 | 0 | 0 | 0 |
| MM 104 roots (healthy) | T | 0 | 0 | 0 | 0 | 0 |
| 1-3B leaves (stem pitting) | 4 | 4 | 4 | 0 | 0 | 0 |
| Seedling leaves (mosaic stem pitting) | 4 | 4 | 4 | 0 | 0 | 0 |
| MM 104 roots (mosaic stem pitting) | 2 | 0 | 0 | 0 | 0 | 0 |

[a] Other controls included: normal serum (1:2), mosaic antiserum (1:2), complement used at 2, 1, and 0.5 full units, respectively. Sparkling hemolysis occurred in the latter controls. No hemolysis occurred in the hemolysin or sheep red blood cell controls.

[b] Rating: 4-equal to the sheep red blood cell control (++++), 2-one half of the red blood cells not hemolyzed (++), T-trace of red blood cells remaining, and 0-sparkling hemolysis.

Table 11. Comparison of Healthy Root and Leaf Antigens vs. Antigens Containing Mosaic and Stem Pitting with Mosaic-Stem Pitting Antiserum and Normal Rabbit Serum.

| Test Antigens (1:5) | Serum Dilutions: Anti-mosaic-stem pitting 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | Normal Rabbit 1:2 | Anticomplementary activity of antigens (1:5)[a] |
|---|---|---|---|---|---|---|---|
| MM 104 leaves (healthy) | 0[b] | 0 | 0 | 0 | 0 | 0 | 0 |
| MM 104 roots (healthy) | T | T | 0 | 0 | 0 | 0 | 0 |
| 1-3B leaves (stem pitting) | 4 | 4 | 2 | T | 0 | 0 | 0 |
| Seedling leaves (mosaic-stem pitting) | 4 | 4 | 4 | 2 | 0 | 0 | 0 |
| MM 104 roots (mosaic-stem pitting) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Other controls included: normal serum (1:2), mosaic serum (1:2), complement used at 2, 1, and 0.5 full units, respectively. Sparkling hemolysis occurred in the latter controls. No hemolysis occurred in the hemolysin or sheep red blood cell controls.

[b] Rating: 4-equal to the sheep red blood cell control (++++), 2-one half of the red blood cells not hemolyzed (++), T-trace of red blood cells remaining, 0-sparkling hemolysis.

Table 12. Comparison of Several Apple Virus Antigens with Mosaic-Stem Pitting Antiserum at a Dilution of 1:4.

| Antigen | Antigen Dilutions | | | | Antigen Anticomplementary Activity |
|---|---|---|---|---|---|
| | 1:5 | 1:10 | 1:20 | 1:40 | 1:5[a] |
| Seedling leaves (mosaic-stem pitting) | 4[b] | 2 | 0 | 0 | 0 |
| 1-3B leaves (stem pitting) | 2 | 2 | 0 | 0 | 0 |
| Virginia Crab Leaves (chlorotic leaf spot) | 0 | 0 | 0 | 0 | 0 |
| Quince leaves (stem pitting) | 4 | 4 | 2 | 0 | 0 |
| Quince leaves (no pitting) | T | T | 0 | 0 | 0 |
| R-12740-7A leaves (dapple apple) | 0 | 0 | 0 | 0 | 0 |
| M. platycarpa leaves (dapple apple) | 0 | 0 | 0 | 0 | 0 |

[a] Other controls included: anti-mosaic-stem pitting serum (1:4), complement used at 2, 1, and 0.5 full units, respectively. Sparkling hemolysis occurred in the latter controls. No hemolysis occurred in the hemolysin or sheep red blood cell controls.

[b] Rating: 4-equal to the sheep red blood cell control (++++), 2-one half of the red blood cells not hemolyzed (++), T-trace of red blood cells remaining, 0-sparkling hemolysis.

Several antigens prepared by the acetone precipitation-tap water dialysis technique from known indexed material were tested with the mosaic-stem pitting antiserum at a dilution of 1:4 (Table 12). No complement fixation occurred with the chlorotic leaf spot infected Virginia Crab or dapple apple leaf antigens from M. platycarpa and R-12740-7A. The quince leaf antigen inoculated with stem pitting gave a ++++ reading when used at a dilution of 1:10. Some trace activity was obtained with the quince leaf antigen which had been inoculated with non-pitted Virginia Crab. Both quince antigens were very viscous and contained a large amount of pectin-like substance. All the above antigens had been stored in sealed ampules at dry ice temperature for 15 days.

The mosaic-stem pitting inoculated seedling and the 1-3B antigens also resulted in complement fixation but the degree of activity was less than in previous tests, possibly due to the storage of these 2 antigens for 27 days at 4° C.

B. Dapple Apple Antisera Tests. Antiserum obtained from subcutaneous injections of the dapple apple infected R-12740-7A leaves was found to be active with several test antigens. It reacted with the homologous R-12740-7A test antigen as well as with a dapple apple antigen prepared from leaves of inoculated M. platycarpa (Table 13).

Apple leaf antigens of M. platycarpa inoculated with green mottle and russet ring also exhibited complement

fixing activity with the dapple apple antiserum. The latter 2 virus isolates cause ring patterns on apple fruits, as does the dapple apple isolate.

Data in Table 13 also indicate that the healthy MM 104 leaf antigen used at a dilution of 1:2 showed some complement fixing activity, although this antigen had demonstrated no activity with the earlier mosaic-stem pitting antiserum.

Table 13. Comparison of Several Apple Virus Antigens with Dapple Apple Virus Antiserum at a Dilution of 1:4.

| Antigens | Antigen Dilutions | | | Anticomplementary activity of antigens (1:2)[a] |
|---|---|---|---|---|
| | 1:2 | 1:4 | 1:8 | |
| *M. platycarpa* leaves (dapple apple) | 4[b] | 2 | T | 0 |
| R-12740-7A leaves (dapple apple) | 4 | T | 0 | 0 |
| *M. platycarpa* leaves (green mottle) | 4 | 2 | T | 0 |
| *M. platycarpa* leaves (russet ring) | 4 | 2 | T | 0 |
| MM 104 leaves (healthy) | T | 0 | 0 | 0 |

[a] Other controls included: dapple apple serum (1:4), complement used at 2, 1, and 0.5 full units, respectively. Sparkling hemolysis occurred in the latter controls. No hemolysis occurred in the hemolysin or sheep red blood cell controls.

[b] Rating: 4-equal to the sheep red blood cell control (++++), 2-one half of the red blood cells not hemolyzed (++), T-trace of red blood cells remaining, 0-sparkling hemolysis.

## 6. Virus Inactivation

A. Hot Water Treatments. Attempts to obtain budwood free from stem pitting through the use of hot water therapy were not successful. None of the budwood sticks that received a 20 minute exposure at water bath temperature of 50° C. produced viable buds. In general, the buds farthest from the shoot apex withstood the 50° C. temperatures for longer periods of time than did those taken from the top 1/4th of the budsticks (Table 14).

Table 14. Heat Therapy of Stem Pitting Infected Budwood Utilizing a Water Bath Temperature of 50° C. for Periods of 5, 10, and 15 Minutes.

| Min. at 50°C. | Location of Bud on Scion: Apex to Base | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | |
| | Bud Growth | Pitted | Bud Growth | Pitted | Bud Growth | Pitted | Bud Growth | Pitted |
| 0 | 4[a] | 4 | 5 | 5 | 5 | 5 | 5 | 5 |
| 5 | 0 | 0 | 5 | 5 | 3 | 3 | 5 | 5 |
| 10 | 0 | 0 | 0 | 0 | 1 | 1 | 4 | 4 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Number of buds that survived the heat treatment and produced shoots; 2 replicates repeated 4 times for a possible 8 buds.

B. Hot Air Treatments. Three of the 18 Baldwin buds and 4 of the 18 Northern Spy buds subjected to hot air treatments of 37.2 - 38.3° C. survived and produced shoot growth.

Five buds from a single Baldwin shoot and 6 buds from a single Northern Spy shoot were indexed for chlorotic leaf spot on M. platycarpa and the R-12740-7A apple indicators. No leaf symptoms were produced on either indicator from the 2 shoots sampled. Greenhouse temperatures that prevailed while the indicators were growing frequently rose above 27° C., and check plants inoculated with chlorotic leaf spot failed to develop leaf symptoms. However, pitting was observed on the R-12740-7A indicators used as checks. When the R-12740-7A indicators, inoculated with buds from the heat-treated shoots, were examined for pitting it was found that all 6 buds from the Northern Spy shoot produced pitting but only 3 of the Baldwin heat-treated shoot buds had indexed positive for stem pitting. No stem pitting was observed on any of the M. platycarpa indicators.

C. Irradiation of Dormant Scions. Attempts to inactivate stem pitting and dapple apple virus by irradiating dormant budwood with cobalt 60 were unsuccessful. Although 70% of the untreated checks made unions with the seedling rootstocks and produced shoots, a 100% lethality of the 180 grafts from the irradiated treated dormant scions resulted. Information received from British Columbia (1961) during the course of the experiment confirmed that the minimum gamma ray irradiation of 25,000r used in attempting to obtain virus free budwood was above the lethal dosage. In British Columbia tests, 4500r to 5000r of gamma rays was found to produce about 50% lethality of dormant apple scions.

# SECTION V

## DISCUSSION

One of the problems in selecting Malus host indicators for apple virus diseases is to secure plants that are free from latent viruses. Several indicators which had been collected for use in this study were found to be carrying latent virus infections. Some of these species had been used previously as indicators by other workers. Since strains of virus are known to vary in virulence, the use of an indicator which contains a non-virulent strain may preclude symptom development when inoculated with isolates carrying a severe strain. Cross indexing of indicators resulted in reducing the total number of indicators suitable for use in determining relationships of apple virus diseases. Attempts to find additional seedling clones resulted in the selection of 2 apple clones which may offer promise in future indexing as indicators for mosaic and stem pitting.

Several virus isolates were found to contain more than one virus entity. Flat limb, for example, produced reactions on all indicators used in this study. In order to determine which virus or combination of viruses is responsible for flat limb, separation of the virus components is necessary. Passage of this isolate through filtering hosts would offer a possible solution. Another method would

be to inoculate single virus isolates into a virus-free tree in order to determine which virus entity is responsible for the symptom expression known as flat limb. Several isolates were obtained which appear to contain single virus entities and have been used in this manner; however, as flat limb has a long incubation period, no definite results have been obtained to date.

In some cases the degree of symptom reaction in an indicator can be correlated with the number of virus entities used as inoculum. Both the Malus platycarpa indicator and Virginia Crab showed reductions in total growth commensurate with the number of virus factors contained in the inoculum source. It is not unlikely that latent viruses also produce a similar reaction in commercial bearing trees.

Under field conditions the incubation period for rubbery wood symptom expression in the indicator Lord Lambourne was found to take at least 3 years from the time of initial inoculum. Dieback of terminal shoots of Lord Lambourne prevented the use of 2-year old whips for symptom development. Whether this was due to the rubbery wood virus factor or resulted from additional factors is not known. The presence of 2-year old side shoots was necessary before rubbery wood expression developed. Consequently, virus isolates indexed on Lord Lambourne will need an additional year's incubation period before any relationship to rubbery wood can be demonstrated. That stem pitting does not appear to be related to rubbery wood is suggested by the fact that

2 Lord Lambourne indicator clones not showing rubbery wood have indexed positive for stem pitting.

The chlorotic leaf spot factor was transmitted by bud inoculation in one case, even though bud union was not successful. Stem pitting, however, was not transmitted under such circumstances. A bud union of over 2 weeks was found to be necessary before stem pitting was transmitted. The apparent short infection period necessary for chlorotic leaf spot might serve to explain why this virus is found to be prevalent in such a high degree in commercial varieties. Mechanical transmission of this virus may be found to occur during the course of budding and grafting operations in some cases, as sap transmission of this virus to herbaceous hosts has been reported.

Since many of the isolates used were found to index positive on all indicators, relationships employing host range studies were disappointing. Certain isolates, such as the 1-12A which pitted Virginia Crab but produced no symptoms on the R-12740-7A, further confirms that these 2 disease expressions have different causal agents. The reverse situation, in which the chlorotic leaf spot isolate did not cause pitting in Virginia Crab, supports this conclusion. Information of this type points out that Virginia Crab should be included as an indicator host when searching for apple material that does not contain the stem pitting virus entity.

Dapple apple, which occurred independently of stem pitting and has been found on non-pitted Virginia Crab trees, did not produce pitting in Virginia Crab and it is thus considered not to be related to stem pitting. Results obtained with the serological tests offer further support in this regard.

Quince mosaic which produced definite mosaic symptoms on apple seedlings did not seriously affect the R-12740-7A indicator and would have been overlooked if a detailed search for mosaic symptoms had not been made on this inoculated host. Although it is not known whether this isolate is common to other strains of apple mosaic, the reaction of the R-12740-7A indicator to this inoculum may indicate that in some cases when apple mosaic occurs apart from other viruses it may escape detection in this indicator.

Indicator behavior to dapple apple was unusual in that symptoms were observed only in the R-12740-7A indicator. All other virus isolates that initiated symptoms in the latter indicator also indexed positive for line pattern in M. platycarpa. The dapple apple isolate may also carry another virus entity which is responsible for chlorotic leaf spot on R-12740-7A, for it was found that the scar skin isolate, which is also a fruit distorting entity, did not produce symptoms on any of the indicators used. Scar skin does not appear to be related to stem pitting since it did not pit Virginia Crab. The fact that it did not produce

symptoms on any of the indicators points out the need for a certification program of apple budwood which will include means to identify and prevent the spread of viruses which are potentially damaging to fruit. It also indicates the complexity of establishing a standard list of apple indicators.

The symptom referred to as dieback, produced when 1 and 2-year old indicators of R-12740-7A were budded directly, was noticed only when the inoculum source was known to contain stem pitting. Frequently when this indicator was used in double budding index tests, virus inoculum resulted in extreme stunting of the indicator and, in some cases, shoot development did not take place. When R-12740-7A was used in greenhouse tests where high temperatures often prevailed, leaf symptoms were prevented from developing. High temperatures, however, did not suppress the development of stem pitting in this indicator. Therefore, R-12740-7A would appear to be a good indicator where indexing is attempted in warmer climes, or where temperatures above 70° F. are experienced frequently.

Attempts to inactivate virus in apple budwood were discouraging. Some indications suggest that dapple apple may be inactivated by extended periods of 98-101° F. air temperature. Hot air therapy coupled with meristematic tip propagations might further enhance successful attempts at obtaining virus free material.

Production of antisera active to the homologous

immunizing antigen and to other virus isolates was demonstrated in complement fixation tests. Although the titres of antisera and antigen preparations were low, such titres are not as important in complement fixation reactions as they would be in precipitation reactions where considerable amounts of antigen are needed to produce visible results. Titres of 1:32 with the dapple apple preparations, and of 1:80 for stem pitting, based on initial dilutions of infected leaf tissue, were obtained. The presence of common antigens in green mottle and russet ring isolates obtained with dapple apple antisera indicates that the 3 virus diseases may be caused by the same virus, or by strains of the same virus.

Usually positive reactions for complement fixation tests are rated as 0, +, ++, +++, and ++++, indicating the degree of red blood cells lysed, ie, 100%, 75%, 50%, 25%, and 0.0% equivalent to the red blood cell control. However, in tests conducted in this study the visual positive ratings were not so finely divided since the original objective was to see if a serological approach using complement fixation was possible. Granting that serologically active preparations are not indicative of infective virus, inasmuch as serological activity is dependent only on the virus protein fraction, methods which increase the amounts of serologically active virus in general may be correlated with increased amounts of infective virus. This, of course, can be checked with chlorotic leaf spot and the Tulare

strain of apple mosaic where herbaceous indicators are available for infectivity studies. Production of active antisera will furnish a means of comparing serological relationships of apple viruses to other plant viruses. Simply because a virus produces visible symptoms in apples does not mean that it is endemic to that host alone, and it is to be expected that some "apple" viruses will be found that are synonymous with presently known viruses for which considerable information may already exist. Some of the so-called latent apple viruses which appear to cause no visible damage to commercial apple varieties may be found to be responsible for serious damage in other plants. In instances where this is found to exist, latent virus infection in apples constitutes a potentially dangerous situation, particularly so if the virus is transmitted by vectors.

# SECTION VI

## SUMMARY

A comparison of apple virus isolates employing indicator host plants and complement fixation tests revealed that Virginia Crab stem pitting is not related to dapple apple, chlorotic leaf spot, rubbery wood or scar skin virus disorders. Attempts to demonstrate virus relationships among stem pitting, russet ring, green mottle and flat limb infected trees were complicated by the presence of contaminating latent viruses which occurred in the latter 3 isolates. The flat limb isolate collected from Nova Scotia gave a positive reaction for rubbery wood in the indicator variety Lord Lambourne. Flat limb induced 6 other distinct symptom expressions on apple indicators. It was found in field tests that an incubation period of at least 3 years from time of initial inoculation was required for rubbery wood symptom expression in the indicator Lord Lambourne. Quince (Chaenomeles sp.) was found to be a host for stem pitting virus. Apple virus isolates that contained stem pitting as a single entity did not produce chlorotic leaf spot when indexed on R-12740-7A. The dapple apple isolate was unusual in that it was the only isolate which indexed positive on R-12740-7A for chlorotic leaf spot but did not cause line pattern or other symptoms in Malus platycarpa (Long Ashton). An apple virus isolate

designated as "scar skin" indexed negative on Virginia Crab, R-12740-7A and Malus platycarpa indicators. The degree of symptom reaction observed in apple indicators was, in general, found to be correlated with the number of latent virus entities contained in the inoculum source.

Antisera to dapple apple and a mosaic-stem pitting virus complex were obtained when apple root and leaf tissues were used as immunizing antigen sources. Infected tissues were prepared by grinding in liquid nitrogen and then homogenizing the powder in 0.04M pH 7.2 phosphate buffer containing 0.04M sodium diethyldithiocarbamate and 0.01M thioglycolic acid. Following low speed clarification, the supernatant was subjected to high speed centrifugation. The resulting pellet was resuspended in the original buffered solution and homogenized with a Drakeol 6-VR:Arlacel A (9:1) adjuvent and injected into rabbits subcutaneously. Antigenic preparations that were not anticomplementary were obtained by a nicotine acetone-precipitation tap water-dialysis procedure. Complement fixation tests revealed the presence of common antigens in green mottle, russet ring, and dapple apple virus isolates.

## SECTION VII

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# APPENDIX

## Materials and Amounts Used in Complement Fixation Tests

### Hemolysin Titration

| | Tube No. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Hemolysin (0.1 ml.) | 1000 | 2000 | 3000 | 4000 | 5000 | 6000 | 7000 | -- |
| Saline (0.85%) | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.5 |
| Complement (1:30) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | -- | -- |
| Sheep Red Blood Cells (2%) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Hemolysin dilutions:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.1 ml. | undiluted | plus | 9.9 ml. | saline | -- | 10 ml. | (1:100) |
| 0.2 " | (1:100) | " | 1.8 " | " | -- | 10 " | (1:1000) |
| 0.2 " | (1:1000) | " | 0.2 " | " | -- | 0.4 " | (1:2000) |
| 0.1 " | (1:1000) | " | 0.2 " | " | -- | 0.3 " | (1:3000) |
| 0.1 " | (1:1000) | " | 0.3 " | " | -- | 0.4 " | (1:4000) |
| 0.1 " | (1:1000) | " | 0.4 " | " | -- | 0.5 " | (1:5000) |
| 0.1 " | (1:1000) | " | 0.5 " | " | -- | 0.6 " | (1:6000) |
| 0.1 " | (1:1000) | " | 0.6 " | " | -- | 0.7 " | (1:7000) |

Unit of lysin is smallest amount of lysin producing complete lysis of 0.1 ml. of 2% sheep red blood cells in the presence of excess $C^1$.

## Materials and Amounts Used in Complement Fixation Tests (Cont.)

### Complement Titration

| | Tube | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Hemolysin (2 units) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | -- | -- | 0.1 |
| Saline | 0.3 | 0.25 | 0.2 | 0.15 | 0.1 | 0.05 | -- | 0.5 | 0.1 | 0.4 |
| $C^1$ (1:30) | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 | -- | 0.4 | -- |
| SRBC (2%) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Unit of complement is the smallest amount of complement causing complete lysis of the 0.1 ml. 2% SRBC sensitized with 2 units of lysin.

$$\frac{\text{Dilution of titration (30)}}{\text{Volume in titration}} = \frac{X}{\text{Volume needed in test}}$$

Materials and Amounts Used in Complement Fixation Tests (Cont.)

Standard Controls

| | Antigen | Serum | $C^1$ | NaCl | Hemolysin | SRBC |
|---|---|---|---|---|---|---|
| 1. Antigen | 0.1 | -- | 0.2 | 0.1 | 0.1 | 0.1 |
| 2. $C^1$ (0.5 FU) | -- | -- | 0.05 | 0.35 | 0.1 | 0.1 |
| 3. $C^1$ (1 FU) | -- | -- | 0.1 | 0.3 | 0.1 | 0.1 |
| 4. $C^1$ (2 FU) | -- | -- | 0.2 | 0.2 | 0.1 | 0.1 |
| 5. Hemolysin | -- | -- | -- | 0.4 | 0.1 | 0.1 |
| 6. SRBC | -- | -- | -- | 0.5 | -- | 0.1 |
| 7. Serum | -- | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 |

Test Procedure

| Antigen Dilutions | Antigen | Serum | $C^1$ (2 FU) | NaCl | Hemolysin (1:1000) | SRBC |
|---|---|---|---|---|---|---|
| (1:2) | 0.1 | 0.1 | 0.2 | -- | 0.1 | 0.1 |
| (1:4) | 0.1 | 0.1 | 0.2 | -- | 0.1 | 0.1 |
| (1:8) | 0.1 | 0.1 | 0.2 | -- | 0.1 | 0.1 |

Actual Tube Test Dilutions in Ml. (Table 10)

| Tube No. | Antigens (1:5) | | Sera | $C^1$ 1:20 (2 FU) | Hemolysin (1:1000) | SRBC 2% | NaCl 0.85% | Results |
|---|---|---|---|---|---|---|---|---|
| | | | SPV (1:2) | | | | | |
| 1. | H2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | -- | 0 |
| 2. | H1 | " | " | " | " | " | " | 0 |
| 3. | L2 | " | " | " | " | " | " | ++++ |
| 4. | L1 | " | " | " | " | " | " | ++++ |
| 5. | RE | " | " | " | " | " | " | ++++ |
| | | | SPV (1:4) | | | | | |
| 6. | H2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | -- | 0 |
| 7. | H1 | " | " | " | " | " | " | 0 |
| 8. | L2 | " | " | " | " | " | " | ++++ |
| 9. | L1 | " | " | " | " | " | " | ++++ |
| 10. | RE | " | " | " | " | " | " | 0 |
| | | | SPV (1:8) | | | | | |
| 11. | H2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | -- | 0 |
| 12. | H1 | " | " | " | " | " | " | 0 |
| 13. | L2 | " | " | " | " | " | " | ++++ |
| 14. | L1 | " | " | " | " | " | " | ++++ |
| 15. | RE | " | " | " | " | " | " | 0 |
| | | | Normal (1:2) | | | | | |
| 16. | H2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | -- | 0 |
| 17. | H1 | " | " | " | " | " | " | 0 |
| 18. | L2 | " | " | " | " | " | " | 0 |
| 19. | L1 | " | " | " | " | " | " | 0 |
| 20. | RE | " | " | " | " | " | " | 0 |
| | | | Normal (1:4) | | | | | |
| 21. | H2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | -- | 0 |
| 22. | H1 | " | " | " | " | " | " | 0 |
| 23. | L2 | " | " | " | " | " | " | 0 |
| 24. | L1 | " | " | " | " | " | " | 0 |
| 25. | RE | " | " | " | " | " | " | 0 |
| | | | Controls | | | | | |
| 26. | H2 | 0.1 | -- | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 27. | H1 | 0.1 | -- | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 28. | L2 | 0.1 | -- | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 29. | L1 | 0.1 | -- | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 30. | RE | 0.1 | -- | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 31. | SPV Serum (1:2) | | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 32. | Normal " " | | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0 |
| 33. | $C^1$ 2 FU | | ---------- | 0.2 | 0.1 | 0.1 | 0.2 | 0 |
| 34. | $C^1$ 1 FU | | ---------- | 0.1 | 0.1 | 0.1 | 0.3 | 0 |
| 35. | $C^1$ .5 FU | | ---------- | 0.05 | 0.1 | 0.1 | 0.35 | 0 |
| 36. | Hemolysin | | --------- | -- | 0.1 | 0.1 | 0.4 | ++++ |
| 37. | SRBC | | ---------- | -- | -- | 0.1 | 0.5 | ++++ |