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Guidelines for Diagnostic Work in Plant Virology

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Foreword

This handbook is intended as a general guide to diagnostic work in plant virology. It is addressed primarily to the extension worker, and is meant to give only the most basic background information on plant viruses, their identification, and the diseases they cause. Those seeking more details should consult some of the texts and journals listed in the bibliography.

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TABLE OF CONTENTS

I. Symptoms of Virus Diseases	1
A. General	1
B. General Appearance	1
C. Color Deviation	1
(1) Leaves	1
(2) Flowers	2
(3) Fruits	2
(4) Roots	2
D. Malformations	3
(1) Leaves	3
(2) Flowers	3
(3) Fruits	3
(4) Stems	3
(5) Roots	3
E. Other Symptoms	3
F. Masking of Symptoms	4
G. Tolerance	4
H. Mixed Infections	4
I. Phenomenon That Cause Symptoms Resembling Those of Virus Infections	4
II. Transmission of Viruses	5
A. Sap Transmission	5
(1) Selection of indicator hosts	5
(2) Preparation of inoculum	6
(3) Procedure for Mechanical Inoculation	7
(4) Symptom Development and Recording	9
B. Transmission by Grafting	9
(1) General	9
(2) Standard grafting methods	9
C. Transmission by Dodder	10

111

D.	Transmission by Insects, Mites, and Nematodes	11
	(1) General	11
	(2) Aphid transmitted viruses	16
	(3) Whitefly transmitted viruses	18
	(4) Leafhopper and planthopper transmitted viruses	20
	(5) Beetle transmitted viruses	22
	(6) Mealybug transmitted viruses	22
	(7) Psyllid transmitted viruses	22
	(8) Thrips transmitted viruses	23
	(9) Mite transmitted viruses	23
	(10) Nematode transmitted viruses	24
III.	Identification of Viruses	25
A.	Determination of Size and Shape	25
	(1) Materials needed	25
	(2) Sample preparation	26
	(3) Electron microscopic examination	27
B.	Determination of the Physical Properties of the Virus	28
	(1) Thermal inactivation point (TIP)	28
	(2) Longevity <u>in vitro</u> (LIV)	28
	(3) Dilution end point (DEP)	28
C.	Determination of Host Range	29
D.	Determination of Insect Vectors	29
E.	Determination of Virus Identity by Serological Methods	29
IV.	Storage of Viruses	31
V.	Control of Virus Diseases	32
A.	Preventing Infection	32
B.	Minimizing the Spread Within a Crop	32
C.	Using Healthy Planting Materials	32
D.	Cross Protection	34
VI.	Useful References	35

I. Symptoms of Virus Diseases

A. General

Field diagnosis based on symptoms alone should only serve as a guide. Symptoms can provide only a partial diagnosis.

- Similar symptoms can be produced by different viruses.
- The same virus can produce a range of symptoms, depending on environment and host genotype.
- A lack of symptoms does not necessarily mean that no viruses are present. It may simply mean that the infection is latent.

B. General Appearance

- abnormal color
- dwarfing
- stunting (often one-sided)
- rosetting (shortening of the internodes, which produces a bunched appearance)
- witches' broom (excessive budding and branching, stunting, and shortening of internodes)
- decline (loss of vigor)
 - of the whole plant
 - of parts of the plants

C. Color Deviation

(1) Leaves

(a) Discoloration evenly distributed:

- chlorosis (weakening of the green color)
- bleaching (disappearance of all color, white appearance)
- yellowing (chlorosis and dominance of yellow pigments)
- reddening (abnormal anthocyanin formation)
(caution: can also be caused by mineral deficiencies)

11

- browning and blackening (production of dark melanin-like substances)
- bronzing (necrosis and collapse of epidermal cells covering the still green and apparently healthy mesophyll) –caution: can also be caused by mites

(b) Discoloration irregularly distributed:

- mosaic (pale green, yellow or chlorotic areas, sharply bordered by small veins that are often angular in appearance)
- mottle (discolored areas of various rounded shapes, often diffusely bordered)
- local lesions ranging in size from small pinpoint-size chlorotic or necrotic areas to large irregular patches
- ringspots (single or concentric rings of chlorotic or necrotic tissue separated by normal green tissue)
- streaking (elongated, sharply defined chlorotic patches)

(c) Certain leaf parts uniformly discolored:

- vein yellowing (yellow discoloration of the veins due to lack of chlorophyll; accented color of carotenes and xanthophylls)
- vein clearing (veins appear translucent rather than chlorotic or yellow)
- vein banding (discolored areas along the veins)
- vein necrosis (death of vascular tissues resulting in their turning brown)

(2) Flowers

- phyllody (floral parts develop like normal foliage)
- color deviation (intensification, weakening or change of pigments in the epidermal layer of the petals)
- breaking (usually consists of flecks, streaks or sectors of abnormally colored tissue. Caution: may be confused with genetic variegation)
- virescence (general greening of the petals)

(3) Fruit discoloration:

- of the whole fruit
- of parts of the fruit (marbling, mottling, spotting)

(4) Roots

- lesions
- necrosis

D. Malformation

(1) Leaves

- distortion (crinkling, curling, twisting)
- epinasty (curling downwards)
- narrowing (reduction of laminar tissue; vein growth remains almost normal)
- size reduction
- thickening
 - of all or part of the lamina
 - of the veins
- enations (outgrowths of the leaf blade, often resulting in curling of leaves)

(2) Flowers

- various kinds of distortions
- abnormal flower parts

(3) Fruits

- deformation and irregular shapes
- tumorous swellings
- abortive seeds

(4) Stems

- distortion
- shortening of internodes

(5) Roots

- decay and dieback
- tumors

E. Other Symptoms

- wilting
- defoliation
- premature leafdrop
- deviation in flower number
- premature or delayed flowering
- abnormal fruit flavor
- abnormal secretion
- gummosis
- bark scaling
- wood pitting
- shoot swelling
- graft incompatibility

F. Masking of Symptoms

Under certain environmental conditions no visible symptoms are produced, even though virus is present in the plant. This is also referred to as a latent infection, and is generally due to environmental factors such as temperature, light, and nutrient excesses or deficiencies.

G. Tolerance

Due to the genetic disposition of the plant, no visible symptoms are produced by the presence of the virus.

H. Mixed infections

Symptoms are caused by several viruses infecting one plant.

I. Phenomena that cause symptoms resembling those of virus infections:

- genetic abnormalities
- nutritional deficiencies
- herbicide damage
- insect or mite damage
- air pollution damage

Symptoms caused by these agents are neither sap nor graft-transmissible, and recovery is common.

II. Transmission of Viruses

A. Sap Transmission (mechanical inoculation)

Sap transmission is the application of virus-containing plant extracts (i.e. inoculum) to the leaf surface of healthy plants.

In order for the virus particles to penetrate the cuticle and epidermis of a healthy leaf, the surfaces must be artificially wounded. When the inoculated plant is susceptible, the following reactions may occur:

- Local lesions on the inoculated leaves
- Systemic symptoms (mottle, mosaic, leaf deformation, local lesions, necrosis, etc. that are distributed throughout the plant)
- No symptoms:
 - (a) Although the virus has invaded the plant and is multiplying, no host reaction is visible. Either the plant is tolerant of the virus or the symptoms are masked by environmental conditions.
 - (b) Although the virus has entered the plant, it is not multiplying and invading other parts of the plant, and no symptoms are produced. The plant is resistant to the virus.
 - (c) The virus has not entered the plant; the plant is immune to the virus.

Note: Not all viruses can be transmitted mechanically. Viruses that in nature persist in the vector. (semipersistent and persistent aphid-transmitted viruses, as well as the leafhopper and whitefly transmitted viruses) are not usually transmitted by sap.

(1) Selection of Indicator Hosts

Indicator hosts react diagnostically to certain viruses. They can be used to distinguish between these viruses, usually by observing immunity to one and susceptibility to the other.

The most commonly used indicator plants are:

- Chenopodium amaranticolor (susceptible to more than 40 different viruses)
- Chenopodium quinoa
- Cucumis sativus
- Datura stramonium
- Gomphrena globosa
- Nicotiana benthamiana
- Nicotiana glauca

- Nicotiana tabacum 'Xanthi'
- Nicotiana tabacum 'Samsun'
- Phaseolus vulgaris 'Pinto'
- Vicia faba
- Vigna unguiculata

(a) Seeds

Seeds of indicator plants can be obtained from:

Plant Introduction
Germplasm Resources Laboratory
Agricultural Research Center
Beltsville, MD. 20705
USA

A small number of seeds should be obtained initially. Propagation should be carried out in an insect-proof greenhouse.

(b) Illumination

Reduced light intensity is known to increase the susceptibility of some plants to certain viruses. Keeping indicator plants in the dark for several hours or days prior to inoculation may increase their susceptibility.

(c) Soil

To inactivate microbial pathogens and soil-inhabiting viruses and virus vectors, the soil should be steam-sterilized at 100°C for 30 minutes.

(d) Greenhouse

- Indicator plants must be kept in an insect-free greenhouse or screenhouse.
- Healthy plants should be kept isolated from inoculated plants, preferably in a separate room.
- To avoid a buildup of insects, the greenhouse should be sprayed regularly with an insecticide.

(2) Preparation of Inoculum

Inoculum is the sap extracted from diseased plants for use in transmitting the virus.

The following points should be kept in mind when choosing virus-infected leaf tissue for inoculum preparation:

- Virus content does not always correlate with the severity of the symptoms.
- The highest virus content is often found in young tissues.
- Some viruses can only be transmitted at certain times of the year.

(a) Maceration of virus infected tissue

One part virus infected tissue is ground up in a small mortar with 2 to 5 parts buffer, generally 0.01 M phosphate buffer, pH 7.0.

The inoculum should be kept cool and used immediately.

The buffer is prepared in the following way:

Solution A: 1.36 g KH_2PO_4 in 1000 ml H_2O

Solution B: 1.78 g $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$ in 1000 ml H_2O

51.0 ml of solution B mixed with 49.0 ml of solution A gives 100 ml of 0.01 M phosphate buffer solution pH 7.0.

Reference: Noordam, 1973.

(b) Inoculum additives

- Abrasives

The use of abrasives increases infection by providing wounds for the entry of virus particles. The most commonly used are Carborundum (silicon carbide, 400-600 mesh) and Celite (diatomaceous earth). The abrasive is either finely dusted over the leaf surface before inoculation or suspended in the inoculum (0.5-1% w/v).

- Stabilizing additives

Many plants contain inhibitors that may inactivate the virus, decrease or inhibit its infectivity, or interfere with its transmission. The following compounds, when added to the inoculum, are known to have a stabilizing effect on viruses in plant extracts containing such inhibitors. They also have a stabilizing effect on unstable viruses.

- Ethylenediaminetetraacetic acid trisodium salt (EDTA)	0.0005-0.1 M
- Thioglycollic acid (TGA)	0.01-0.1 M
- 2-mercaptoethanol (MCE)	0.015-0.15 M
- Sodium diethyldithiocarbamate (DIECA)	0.01-0.1 M
- Ascorbic acid (Vitamin C)	0.02-0.17 M
- Sodium sulfite (Na_2SO_3)	0.02-0.05 M
- Bovine serum albumine	0.01%

Any of these compounds can be added to the inoculum in the concentration range listed. The selection of the compound and concentration depends on the particular virus/host plant system.

(3) Procedure for Mechanical Inoculation

Routine Inoculation Method:

Grind approximately 5 g of virus infected leaves with 10 to 20 ml phosphate buffer (pH 7.0) in a sterilized mortar. Add EDTA or DIECA as a stabilizing agent. Gently rub the suspension on the leaves of healthy indicator plants which have been dusted with Carborundum. Rinse the leaves with water after the inoculation.

Indicator plants	- Inoculate at least two plants of every species. One control plant of each species should be set aside for later comparison of symptoms.
Pre-inoculation	Keeping indicator plants in the dark for several hours or days prior to inoculation may increase their susceptibility.
Age	- Young plants are generally more susceptible to virus infection than are older plants.
Time	- Plants are generally more susceptible to virus infection in the afternoon.
Inoculation site	<ul style="list-style-type: none"> - Upper leaf surface Peas and beans: Inoculate the primary leaves. Cucumber: Inoculate the cotyledons. Chenopodium: Inoculate the fourth to eighth leaf. Tobacco: Any leaf can be inoculated from the three to four leaf stage. Datura: Inoculate when the first or second leafpair has developed.
Glassware	- Use sterilized glassware; autoclave for 30 minutes at 120°C or boil in water for three hours.
Abrasives	- Either add to inoculum or apply to leaves prior to inoculation.
Application of inoculum	- Apply the inoculum gently to the leaf surface with a cotton swab, a pad of cheesecloth, or a glass rod with a flattened end.
Post-inoculation treatments	<ul style="list-style-type: none"> - Rinsing the inoculated leaves with water is thought to remove natural toxins in the inoculum which interfere with infection, and to reduce injury from chemicals which have been added to the inoculum. It also facilitates later observation of symptoms. - Light reduction Several hours of darkness after

Inoculation can increase the susceptibility of virus indicator plants and promote better symptom expression.

- Quick drying of leaves
This can be done with an atomizer or with blotting paper.

(4) Symptom Development and Recording

- Plants should be observed every day for several weeks (in some cases for several months, e.g. transmission of viruses from woody plants). Compare with control plants of the same age.
- Many host plants will develop local lesions, but other symptoms can also appear.
- Distinguish between local reaction on the inoculated leaves and systemic reaction on the non-inoculated leaves.
- Record symptoms and their sequence.
- Some of the common symbols used for recording symptoms:

LL : local lesions
nLL : necrotic local lesions
cLL : chlorotic local lesions
Vc : vein clearing
M : mosaic
Mo : mottle
N : systemic necrosis
Mal : malformation
E : etching
RS : ringspot

B. Transmission by Grafting

Most viruses can be transmitted by grafting.

(1) General

Materials needed for grafting:

- Sharp razor blade (for soft tissues)
- Sharp knife (for woody tissues)
- Plastic tape (approximately 2 cm wide)

To prevent contamination, the knife or razor blade should be flamed with alcohol before use.

(2) Standard Grafting Methods

(a) Cleft grafts (Fig. 1, a and b)

- Top cleft grafting

This method, which is also called wedge grafting, is widely used with both herbaceous and woody plants.

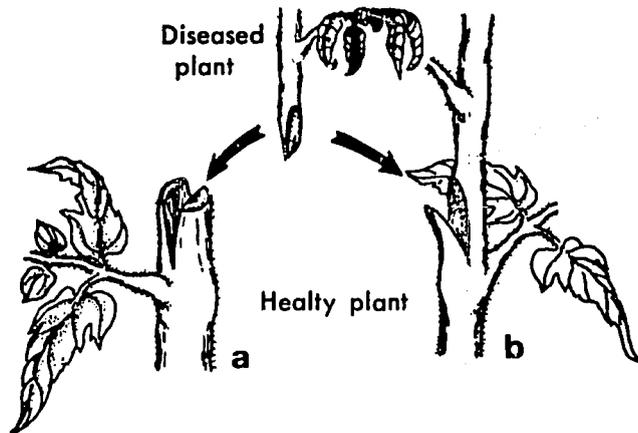


Figure 1. Top Cleft Grafting
b. Side Cleft Grafting

The top of a diseased plant is cut off and a slit is cut axially through the middle of its stem. After its end has been cut into a wedge shape, the top scion from a healthy plant is inserted tightly into this slit, and the joint is then wrapped with plastic tape. Symptoms of systemic infection are observed in the new growth of the originally healthy plant parts. The growing tip of the plants may need to be cut back to promote lateral buds with obvious virus symptoms.

- Side cleft grafting

A cleft is made tangentially in the main stem near one of the leaf nodes. The virus infected scion is inserted into the slit as described above.

(b) Approach graft (Fig. 2)

The stems of a virus-infected and a virus-free plant are cut lengthwise so that the cambium is exposed. Stems of similar thickness are usually chosen. The cut portions are joined, and the union is then wrapped with plastic tape. The growing tip of the healthy plant is cut back to promote the development of lateral buds. If the infection is systemic, virus symptoms will be observed on the previously healthy plant.

(c) Other grafting methods - consult the literature.

C. Transmission by Dodder

Dodder (*Cuscuta* spp) is a semi-parasitic plant which attaches itself to other plants and draws nutrients from them by means of root-like haustoria. Several species of *Cuscuta* are known to transmit viruses. The most common ones are *C. campestris* and *C. subinclusa*. Dodder plants used for transmission work must be grown from seed so that they will be virus-free.

Place the virus-free dodder plant in close contact with the virus-infected plant. The dodder will wrap itself around the stems

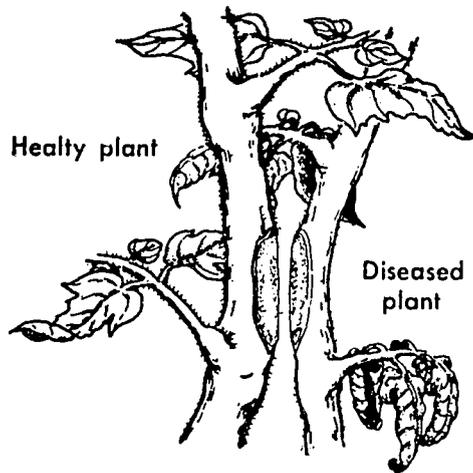


Figure 2. Approach Grafting

and leaves of the virus-infected plant and send out haustoria to form a union with the virus-infected plant. Sap is then passed from one plant to the other. After the dodder has become well established on the diseased plant, its stems can be trained towards the healthy plant. If the virus is transmissible by dodder, virus symptoms will eventually appear on the healthy plant.

D. Transmission by Insects, Mites, and Nematodes

Insect and mite transmission experiments are used to:

- discover the vector of a plant virus.
- assay viruses which are not mechanically transmitted.
- obtain information about the mode of transmission.

The information given below is by no means complete, and should only serve as a guideline in setting up basic transmission work. Some major characteristics of the individual insect groups are presented, with special emphasis on their relationship to viruses. An entomologist should be consulted for any in-depth transmission work.

(1) General

(a) Materials needed for insect transmission studies:

Cages

- **Wooden plant cage (Fig. 3a)**
The cage size is approximately 35x35x50 cm. The sides are covered either with fine wire netting (15 mesh/cm) or a saran screen. The top and front door of the cage are covered with a glass plate. For whiteflies use a cage with two wooden side walls. Each wall should have a round access hole approximately 18 cm in diameter, just large enough for a hand to pass through. The whiteflies are prevented from escaping during handling by black cloth tubes attached to the holes at one end and held closed by rubber bands at the other.

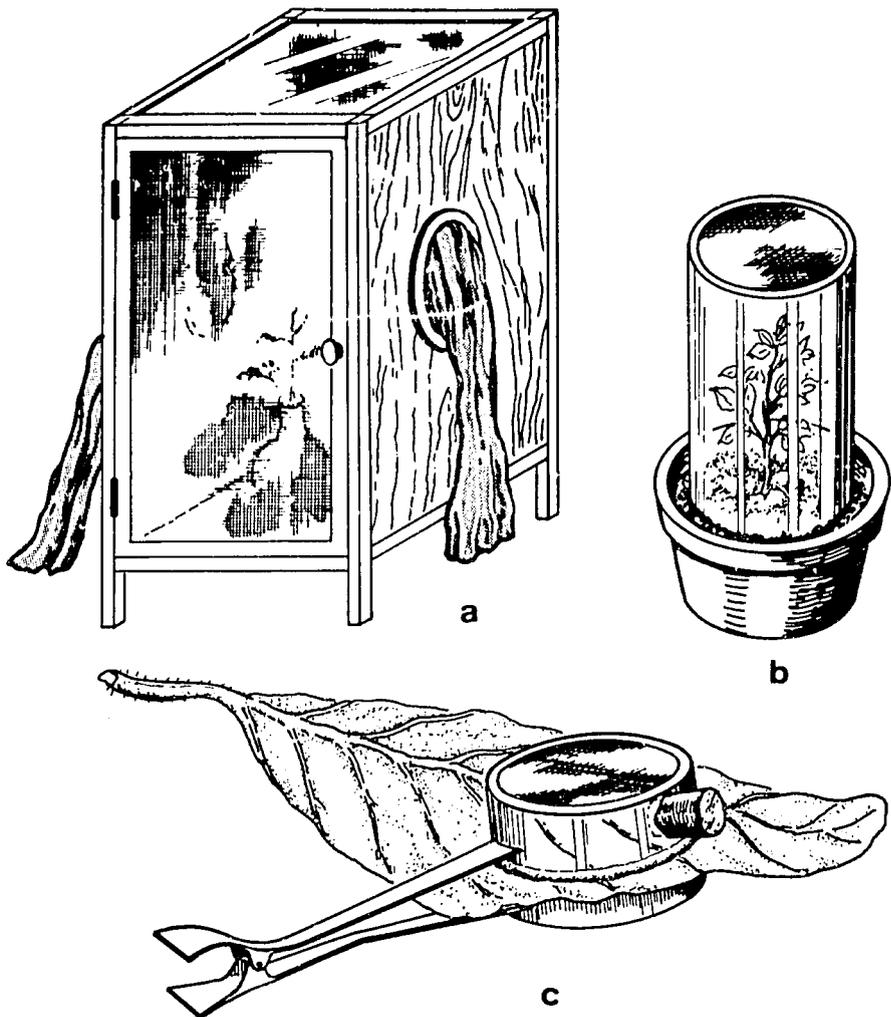


Figure 3. a. Wooden Plant Cage
 b. Plastic Cylinder Whole Plant Cage
 c. Plastic Cylinder Leaf Cage

- Plastic cylinder whole plant cage (Fig. 3b)
 The top of a 13" diameter plastic cylinder is covered with cheesecloth, and the bottom is pressed into the soil of the pot. If a potted plant is not used, fresh leaves in a water-filled test tube can be placed in the plastic cylinder. Cellulose nitrate plastic or butyrate plastic should be used, as certain other kinds (cellulose acetate with diethyl phtaiate) are toxic to plants and insects.
- Plastic cylinder leaf cage (Fig. 3c)
 This kind of cage is used for transmission tests which utilize small numbers of insects, and is made from

sections of plastic tubing approximately 3 cm in diameter and 1.5 cm long, covered on one side with a screen made from a nylon stocking. The insects are transferred through a small hole in the wall of the tube which is then closed with a cork. The cages are attached to the leaves with the aid of hairclips. The hairclips are attached by heating them and pushing them through the wall of the plastic tube.

- Plastic or glass containers
These containers are used to transport insects collected in the field. They should have a screen covering and be large enough to allow for ample space and ventilation.

Insect handling tools

- Artist's brush
The brush is generally used for aphids. The tip should be moistened to make the insect adhere to the brush.
- Aspirator (Fig. 4)
The aspirator is used for more active insects (e.g. leafhoppers and whiteflies). It consists of a small glass bottle closed with a two-hole rubber stopper. A small straight glass tube is inserted through one hole. The outer end is connected to a piece of rubber tubing which serves as a mouthpiece, and the inner end is covered with a small piece of screen. A slightly longer glass tube which has been bent to the desired shape is inserted through the other hole. Insects are sucked into the bottle through this tube.

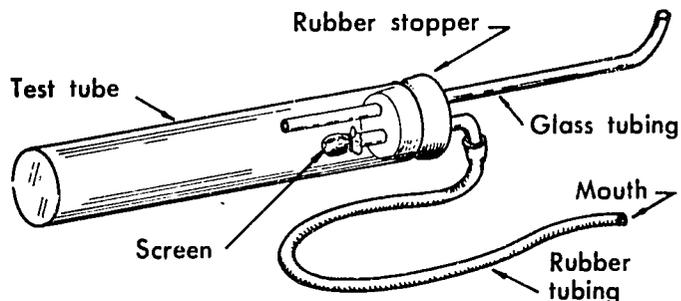


Figure 4. Aspirator

- A single hair
Used for very small insects, e.g. thrips and mites. The hair is fastened to a toothpick or a thin wooden stick.

Test plants

Usually the same plant species are used which the insects were collected from in the field.

(b) Collection of insects in the field

- Sweeping and brushing over low vegetation with a net
- Beating the plant and collecting the fallen insects on a dark sheet spread below
- Collecting individual insects with an artist's brush
- Collecting plant material on which the insect is present
- Trapping insects
 - Color traps - Aphids and whiteflies can be caught in yellow pans filled with water.
 - Light traps - Most insects are attracted to blue-ultraviolet light.
 - Suction traps - Insects are sucked in by a stream of air.
 - Sticky traps - Insects are caught on surfaces covered with a sticky substance. The surfaces are often painted with colors attractive to insects.

(c) Maintenance of insects

- In general, conditions which favor host plant growth also favor the development of vectors. Most vectors can be reared on their host plants or on detached leaves of the same plant.
- Certain insects can be maintained on artificial diets.

Ref: H. L. House, Pritam Singh, and W. W. Batsch. Artificial Diets for Insects: A Compilation of References with Abstracts - Information Bulletin No. 7, Canadian Department of Agriculture, Research Division.

(d) Maintenance of virus-free insect cultures

- Insects collected in the field should be transferred to virus indicator plants to determine whether the insects are virus free.
- Insects should be maintained on host plants which are not susceptible to the virus being studied.
- If the virus is not carried in the vector's eggs (transovarially) the eggs can be used to start a virus-free insect culture. They may be put on wet blotting paper until they hatch. The nymphs can then be transferred to healthy plants.

(e) Inoculation of plants

General procedure:

- Virus-free insects are placed on a virus-infected test plant to feed (acquisition-feeding). Depending on the virus, it may take from a few seconds up to a few days for the insects to become infected. The acquisition period varies with the insect, the virus, and the host plant.
- After the insects have acquired the virus they are immediately transferred to a virus-free test plant for transmission feeding (inoculation feeding). Some insects can transmit the virus immediately, but others can do so only after a latent period, which may vary from a few hours to several weeks. This latent period, i.e. the time between acquisition and transmission, can be determined by successive transfers of the insects to virus-free test plants at hourly intervals after the acquisition feeding .

Some insects, such as certain aphids which carry the virus on their stylet, retain it for as little as 30 minutes. Most leafhoppers (and certain aphids which carry the virus in their gut) are able to transmit the virus throughout their lifetime. Aphids which carry the virus in their haemolymph can also transmit the virus throughout their lifetime, even after molting.

- After the inoculation feeding the insects are usually destroyed with the aid of insecticides or fumigants, and the inoculated plants are observed for the development of typical virus symptoms. Plants should be observed for one to three months.

(f) Control plants and insects

- To check the possibility of the insect culture being infected with virus and to detect virus-like symptoms caused by insect feeding only, some insects should be transferred from the culture plants directly to test plants without feeding on a virus source.
- Insects collected from the field should be transferred to test plants to ensure that they are not already viruliferous.
- Non-inoculated plants should be placed in a greenhouse to detect accidental spread and to ensure that the test plants were not infected before inoculation.

(g) Use of insecticides

Insects will die or develop poorly when put on plants that have been sprayed with insecticides. In cases where insecticides must be applied, use those compounds which are only toxic for a few days.

(2) Aphid Transmitted Viruses

- More than 190 aphid species are known to transmit virus diseases. These are some of the most common virus transmitting aphid species:

<u>Aphis</u> sp.	<u>Myzus</u> sp.
<u>Brevicoryne</u> sp.	<u>Rhopalosiphum</u> sp.
<u>Macrosiphum</u> sp.	<u>Toxoptera</u> sp.

- Aphids are responsible for the transmission of more than 160 different viruses.
- Most aphid-transmitted viruses induce mosaic diseases. Some also produce a yellows-type disease.
- Aphid-transmitted viruses are rarely transmitted transovarially (i.e. through the egg stage). Thus, newly hatched aphids are nearly always virus-free.
- Aphid-transmitted viruses can be grouped into non-persistent (non-circulative), semipersistent, and persistent (circulative) categories. Although most aphid transmitted viruses belong to the first group, some are transmitted atypically, and do not fall in any of these categories. They are transmitted after both short and long acquisition periods, but are not easily transmitted during the period between these two phases. This is referred to as bimodal transmission.

(a) Non-persistent (stylet-borne) viruses

- The virus is acquired by the insect during superficial probing.
- The virus is borne on the stylet, and is usually not ingested.
- The virus is usually retained in the aphid for less than one hour.
- Acquisition access feeding time is short - from a few seconds to a few minutes. Acquisition feeding for a few days is less effective than feeding for a few minutes.
- There is no latent period, and the virus can be transmitted immediately after acquisition access feeding.
- Inoculation feeding time is short - from a few seconds to a few minutes.
- Insects which have fasted prior to acquisition feeding can transmit viruses more effectively.
- Non-persistent viruses are sap-transmissible.
- Non-persistent viruses generally have a wide host range and a low specificity.

- Examples of non-persistent viruses:

bean common mosaic virus
 bean yellow mosaic virus
 cowpea aphid borne mosaic virus
 cucumber mosaic virus
 lettuce mosaic virus
 onion yellow dwarf virus
 papaya ringspot virus
 peanut mottle virus
 pepper mottle virus
 potato virus Y
 soybean mosaic virus
 sugarcane mosaic virus
 tobacco etch virus
 turnip mosaic virus
 watermelon mosaic virus

(b) Semipersistent viruses

- The virus is ingested into the alimentary canal.
- Acquisition access feeding time is somewhat longer than for non-persistent viruses (from several minutes to one or two hours).
- Transmission improves with increased acquisition feeding time.
- There is no latent period in the vector.
- Inoculation feeding is longer than for non-persistent viruses (from several minutes to a few hours).
- Retention in the insect is longer than for non-persistent viruses (12 to 24 hours, and sometimes several days).
- The virus can only be sap transmitted with great difficulty.
- Examples of semi-persistent viruses:

beet yellows virus
 citrus tristeza virus
 clover yellows virus

(c) Persistent (circulative) viruses

- The virus is carried in the haemolymph and in the salivary and alimentary ducts.
- Acquisition time varies from 30 minutes to several hours.
- There is a delay (latent period) before aphids can transmit the virus.
- The efficiency of transmission depends on the amount of virus ingested during acquisition access feeding.

- Transmission occurs only when the inoculation feeding lasts for at least a few hours.
- Fasting has no effect on virus transmission.
- Retention is long, frequently for life. The virus is transstadial, i.e. it is retained through molting.
- Persistent viruses often multiply in the vector (exception: barley yellow dwarf virus).
- Persistent viruses have a narrow to moderate host range, and may be extremely host-specific.
- Persistent viruses are phloem-associated (exception: pea enation mosaic virus).
- Persistent viruses cannot generally be sap-transmitted (exception: pea enation mosaic virus).
- Examples of persistent viruses:
 - barley yellow dwarf virus
 - carrot mottle virus
 - lettuce necrotic yellow virus
 - maize mosaic virus
 - pea enation mosaic virus
 - potato leafroll virus
 - potato yellow dwarf virus
 - rice transitory yellowing virus
 - wheat striate mosaic virus

(d) Bimodally transmitted viruses

- The virus is acquired after short and long acquisition feedings, but is not easily acquired during the interval between these two phases.
- Examples of bimodally transmitted viruses:
 - broad bean wilt virus
 - citrus tristera virus
 - cauliflower mosaic virus
 - dahlia mosaic virus
 - groundnut mosaic virus
 - pea seedborne mosaic virus
 - pea streak virus
 - sweet potato virus A

(3) Whitefly Transmitted Viruses

- Whitefly-transmitted viruses generally cause yellowing, leaf-curling, and some mosaic diseases.
- These viruses are found primarily in tropical and subtropical areas.
- Whitefly-transmitted viruses are persistent in the vector (exception: cucumber vein yellowing virus).

- The virus is carried in the haemolymph.
- A 24 to 48 hour acquisition feeding period on a diseased plant is generally enough to make most whiteflies infective.
- The virus has a variable latent period in the whitefly of 4 to 20 hours.
- The whitefly remains infective for anywhere from a few days to 35 days or longer.
- The virus can be acquired by whitefly larvae. It persists through pupation, and is immediately transmissible by the newly emerged adult.
- There is no evidence of the virus being passed to the egg.
- Whiteflies are phloem feeders.
- Whiteflies prefer to feed on young tissues and on the lower surface of the leaves.
- Whiteflies are carried by the wind, and can spread viruses over great distances.
- Whiteflies are attracted to blue/ultraviolet light and yellow colors.
- Whitefly-transmitted viruses are not transmitted mechanically. (Exceptions: bean golden mosaic virus and tomato golden yellow mosaic virus.)
- Exposure to the viruliferous whitefly vector is considered the most reliable method of screening for resistance to whitefly-transmitted viruses.
- Examples of whitefly-transmitted viruses:
 - abutilon mosaic virus
 - bean crumpling virus
 - bean golden mosaic virus
 - bottle gourd mosaic virus
 - cassava mosaic virus

 - chill leafcurl virus
 - cotton leafcurl virus
 - cucumber vein yellowing virus
 - euphorbia mosaic virus
 - mungbean yellow mosaic virus

 - sweet potato virus B
 - sweet potato mild mottle virus
 - sweet potato stunt virus
 - sweet potato vein clearing virus
 - tobacco leafcurl virus

 - tomato yellow leafcurl virus
 - tomato golden mosaic virus
 - tomato yellow mosaic virus
 - tomato yellow dwarf virus

) Leafhopper and Planthopper Transmitted Viruses

- The most common species of virus-transmitting leafhoppers and planthoppers are:

<u>Agallia</u> sp.	<u>Empoasca</u> sp.
<u>Austrocagallia</u> sp.	<u>Eutettix</u> sp.
<u>Cicadulina</u> sp.	<u>Javesella</u> sp.
<u>Circulifer</u> sp.	<u>Macrosteles</u> sp.
<u>Dalbulus</u> sp.	<u>Nephotettix</u> sp.

- Leafhoppers and planthoppers are phloem feeders; they mainly transmit phloem-associated viruses.
- The viruses are generally transmitted in a persistent (circulative) manner:
 - Their acquisition time varies from 30 minutes to several hours.
 - They have a latent period in the vector.
 - They can only be acquired after an inoculation feeding of several hours.
 - They are retained for life (exception: rice tungro virus).
 - They are carried in the gut and hemolymph.
- The viruses appear to multiply in the vector. (exception: beet curly top virus).
- Transovarial passage occurs with some viruses.
- The viruses have a high vector specificity.
- The viruses have a limited host range.
- Many of the diseases caused by leafhopper-transmitted viruses belong to the yellows and witches' broom disease types.
- The viruses are not sap transmissible (exception: potato yellow dwarf virus).
- Examples of leafhopper transmitted viruses:

barley yellow striate mosaic virus
curly top virus
maize chlorotic dwarf virus
maize streak virus
maize stripe virus
potato yellow dwarf virus
rice tungro virus
rice dwarf virus
rice transitory yellowing virus
rice bunchy top virus
soybean rosette virus

wheat chlorotic streak virus
wound tumor virus

- Examples of planthopper transmitted viruses:

barley yellow striate mosaic virus
cereal tillering disease virus
maize mosaic virus
maize rough dwarf virus
northern cereal mosaic virus
oat sterile dwarf virus
pangola stunt virus
rice black streaked dwarf
rice grassy stunt
rice hoja blanca
rice stripe
stunting of maize
sugarcane Fiji disease virus

(5) Beetle Transmitted Viruses

- Acquisition time is only about five minutes.
- After feeding on infected plants the beetle remains infectious for at least one day, and often much longer.
- The virus is usually carried in the haemolymph. Beetle transmitted viruses are generally stable.
- The viruses can be easily transmitted mechanically.
- Transmission is also possible by maceating the beetle and inoculating plants with the resulting fluid.
- The most common species of virus transmitting beetles are:
flea beetles (Phyllotreta sp.)
mustard beetles (Phaedon sp.)
cucumber beetles (Acalymma sp. and Diabrotica sp.).

- Examples of beetle-transmitted viruses:

Andean potato latent virus
bean pod mottle virus
bean rugose mosaic virus
belladonna mottle virus
broad bean mottle virus
broad bean stain virus
broad bean true mosaic virus
brome mosaic virus
cocoa yellow mosaic virus
cowpea chlorotic mottle virus
cowpea mosaic virus
cowpea severe mosaic virus
eggplant mosaic virus
okra mosaic virus
radish mosaic virus

red clover mottle virus
rice yellow mottle virus
southern bean mosaic virus
sowbane mosaic virus
squash mosaic virus
turnip crinkle virus
turnip rosette mosaic virus
turnip yellow mosaic virus
wild cucumber mosaic virus

(also transmitted by grasshoppers and earwigs)

(6) Mealybug-Transmitted Viruses

The main species of mealybugs known to transmit viruses are:

Planococcus sp.
Pseudococcus sp.
Dysmicoccus sp.

- Mealybugs are often attended by ants. If the ants are controlled, the mealybugs will also be controlled.
- Mealybugs are phloem feeders and feed by sucking.
- Mealybug transmitted viruses are sap transmittable.
- The viruses are semipersistent and possibly stylet-borne.
- The probability of infection increases with the length of the acquisition feeding period. Twenty-four hours is optimum.
- The minimum inoculation feeding time is 15 minutes.
- There is no fasting effect.
- There is no latent period.
- The virus persists through the molt.
- Examples of mealybug transmitted viruses:
 - cacao swollen shoot virus (Only the female mealybugs are vectors.)
 - pineapple latent virus
 - sugarcane spike virus

(7) Psyllid Transmitted Viruses

- The most common species known to transmit viruses are:

Trioza sp.
Diaphorina sp.
Psylla sp.

- The virus is carried in the haemolymph.

- Examples of psyllid-transmitted viruses:

pea red leaf mottle virus
pear leafcurl virus

(8) Thrips Transmitted Viruses

- Thrips require special culturing techniques.
Ref: Sakimura, K. (1961). *Fiant Dis. Reprtr.* 45. p. 76f.
- Thrips usually feed on very young tissue.
- The only known species of virus-transmitting thrips are:

Thrips sp.
Frankliniella sp.

They transmit Tomato spotted wilt virus (TSWV).

- TSWV must be acquired by the larva. The adult then transmits the virus.
- TSWV has a wide host range, and infects at least 166 species of dicotyledons and monocotyledons from 36 families. TSWV is sap transmissible.
- TSWV is persistent in the vector.
- TSWV is very unstable.

(9) Mite Transmitted Viruses

- The most common virus-transmitting mite species are:

Aceria sp.
Brevipalpus sp.
Eryophyes sp.

- The viruses are carried in the alimentary tract.
- They are carried over in the molting.
- They are not passed transovarially to the offspring.
- Transmission improves with longer acquisition feedings.
- Mites are extremely difficult to rear and handle. They must be handled with a single hair. Taffetta must be used for caging.
- Mites prefer to feed on very young plant tissue.
- Extreme care must be taken to avoid confusion between symptoms due to feeding (phytotoxemia) and those due to virus infection.
- Examples of mite transmitted viruses:
agropyron mosaic virus

citrus leprosis virus
 coffee ringspot virus
 fig mosaic virus
 hordeum mosaic virus
 peach mosaic virus
 prunus ringspot virus
 ryegrass mosaic virus
 wheat streak mosaic virus

(10) Nematode Transmitted Viruses

- The three main genera of nematodes known to transmit viruses are:

<u>Trichodorus</u> sp.	(approximately 700 μ length)
<u>Xiphinema</u> sp.	(approximately 6000 μ length)
<u>Longidorus</u> sp.	(approximately 2000 μ length)

- Nematode transmitted viruses are
 - sap transmitted.
 - host specific.
 - lost in molting.
- Viruses are retained in nematodes from a few weeks to several months. Viruses are persistent in Trichodorus sp. and Longidorus sp. for about two weeks, and in Xiphinema sp. for about eight months.
- The probability of transmission increases with the length of acquisition feeding. Forty-eight hours is considered optimal.
- Nematode-borne virus diseases in the field often occur in slowly spreading patches.
- Some examples of nematode transmitted viruses:

pea early browning virus	}	<u>Trichodorus</u> sp.
tobacco rattle virus		

tomato black ring virus	}	<u>Longidorus</u> sp. (Persistence of the virus in the vector is about two weeks)
raspberry ringspot virus		

prunus ringspot virus	}	<u>Xiphinema</u> spp. (Persistence of the virus in the vector is about eight months)
arabis mosaic virus		
cherry leaf roll virus		
cherry rasp leaf virus		
grape fanleaf virus		
peach rosette mosaic virus		
strawberry latent ringspot virus		
tobacco ringspot virus		
tomato ringspot virus		

III. Identification of Viruses

A. Determination of Size and Shape

The size and shape of a virus is determined by electron microscopy. A purified or semi-purified virus preparation is normally used. This requires several cycles of high and low speed centrifugation and subsequent density centrifugation.

Examination from crude sap is also possible, and is described here:

(1) Materials needed:

- Copper grids

Size depends on the electron microscope used. Grids of 3 mm diameter and 150-400 mesh size are the most common. (The mesh size indicates the number of apertures per grid.)

The grids must be coated so that they can support the virus particles. Coating grids requires skill and practice, though, and it is often easier to obtain precoated grids from a virologist at a cooperating institute.

The following materials can be used for coating:

- Collodion 0.2% in amylacetate.
This support film is easy to prepare, but is relatively unstable.
- Formvar 0.2% in 0.5% chloroform or ethylenedichloride.
Although slightly more difficult to prepare, this support film has the advantage of being more stable. It can be stabilized even further by adding carbon, but this can only be done with a special apparatus found in the virology departments of universities and research institutes.
- Fine pointed stainless steel forceps (for manipulating the grids)
- Grid box (for storage and transport of the grids)
- Dust-free double-distilled water
- Chemicals:
Stains:
Uranylacetate (UAC)
Phosphotungstic acid (PTA)

Grid coating chemicals:
Formvar (Polyvinyl formaldehyde)
Collodion (Parlodion)

Electron microscope

(If no electron microscope is available, the specimen grids can be sent for examination to a virologist at a cooperating institute.)

Most of the necessary materials are available from:

LKB Produkter AB
Box 305
S-16126
Bromma, Sweden

or

E. Fulham Inc.
Box 444
Schenectady
New York 12301
U.S.A.

() Sample preparation

(a) Leaf dip method

The leaf dip method is a quick and reliable test for the presence or absence of most viruses in infected plant tissues.

Preparation of the virus infected sample:

- With a razor blade, either cut out a 2x3 mm piece of infected tissue or peel off an epidermal strip from the underside of the infected leaf.
- Using a forceps, dip the tissue sample into the stain drop (2% Potassium phosphotungstate at pH 6.5) for one or two seconds, taking care not to damage the support film on the grid (Fig. 5). (Caution: To prevent dirty preparations, avoid stirring the tissue or dipping it too deeply into the drop.)

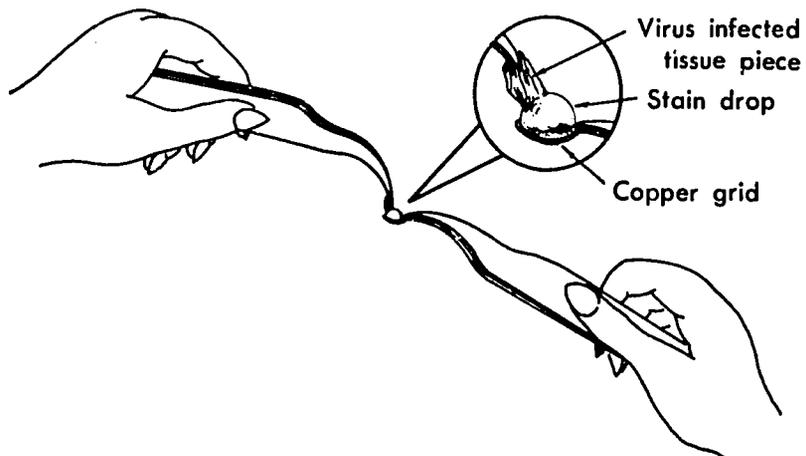


Figure 5. Leaf Dip Method

- It is advisable to make multiple dip preparations with varied dipping times.
- The excess stain can be removed by very lightly touching the edge of the stain drop with a pointed strip of filter paper.

Ref: Brandes, J. (1964). Mitteilungen der Biol. Bundesanstalt für Land und Forstwirtschaft, Berlin-Dahlem, Heft 110, 130 pp.

Hitchborn, J. H. and Hills, G. J. (1965), Virology 27: p. 528-540.

(b) Leaf squash method

This method allows large pieces of the leaf to be used, but leaves large amounts of cell debris on the grid. At times this can make it difficult to detect virus particles in the electron microscope.

- Preparation of the virus infected sample:
 - Place a 1 cm² leaf sample on a clean watch glass or microscope slide.
 - Place 1 to 2 drops of phosphate buffer on the leaf sample and crush it with a glass rod until sufficient cellular material has exuded.
 - Push the crushed leaf remnants to the side and remove the clear sap with a Pasteur pipet.
 - Place one drop of the liquid on a clean parafilm membrane.
 - Place the grid, coated surface downward, on top of the drop. Leave for five minutes.
 - Remove grid with forceps and rinse the coated surface with 40 drops of double-distilled water.
 - Stain with 5 drops of 2% uranylacetate in double-distilled water.
 - Remove excess stain (see leaf dip method).

(3) Electron Microscopic Examination

- Observe the specimen at approximately 32,000 X magnification.
- Photograph it at 5,000 X magnification. (Higher magnification can be achieved with photographic enlargement.)
- Contaminating plant particles such as chloroplast fragments and ribosomes may make it difficult to distinguish virus particles. Clear preparations

may be obtained by grinding up the leaf tissue and passing the sap first through cheesecloth and then through a millipore filter (8 μ opening).

B. Determination of the Physical Properties of the Virus

(1) Thermal Inactivation Point (TIP)

Definition: The TIP is the temperature required to completely inactivate the virus in crude sap during a ten-minute exposure.

Method: Homogenize the infected leaf tissue with a small amount of buffer. Pass the crude sap through cheesecloth. With a pipette add 2 ml of the sap to each of eight screw-capped test tubes, being careful not to let the sap drip along the walls of the test tube. Each tube is heated in a water bath for ten minutes. Preliminary testing should be at 10°C intervals (30°C to 100°C).

After heating, the tubes are cooled immediately in ice cold water. Test plants, preferably those which will react to the virus with local lesion formation, are then inoculated with the samples.

The test plants are observed for symptoms for four days to three weeks, and the temperature range in which virus activity ceases is recorded (e.g. 60-70°C). For determination of the exact TIP, this temperature range is then divided into five smaller intervals (e.g. 59, 62, 65, 68, and 71°C). Five test tubes with sap prepared in the same manner as described above are heated, and test plants are inoculated. The lowest temperature at which no symptoms appear on the inoculated test plants is the TIP.

(2) Longevity In Vitro (LIV)

Definition: The LIV is defined as the length of time the virus is infective in crude sap kept at room temperature (approximately 20 to 22°C).

Method: Use a clarified extract similar to that used to test for TIP, but with 0.01% Streptomycin or Aureomycin added. (These are antibiotics which prevent bacterial contamination.) Ten screw-capped test tubes are each filled with 2 ml of sap. Test plants (preferably local lesion hosts) are inoculated at various time intervals (1, 3, 6, 9, 12, 15, 30, 60, 90, 150 days) and observed for symptom development. If symptoms appear at the 60 day interval but not at the 90 day interval, the LIV is between 60 and 90 days. For an exact LIV determination, time intervals of 2 to 5 days should then be tested within the range of 60 to 90 days.

(3) Dilution End Point (DEP)

Definition: The DEP is the highest dilution of plant sap in which a virus is still infectious.

Method: Homogenize infected leaf tissue in a small amount of buffer. Several dilutions can then be made from this undiluted sap: 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} .

10^{-1} dilution: 1 ml undiluted sap + 9 ml buffer
(Shake well.)

10^{-2} dilution: 1 ml of 10^{-1} dilution + 9 ml buffer
(Shake well.)

10^{-3} dilution: 1 ml of 10^{-2} dilution + 9 ml buffer
(Shake well.)

Use a similar procedure to make additional dilutions.

Inoculate hosts, preferably local lesion hosts, with undiluted sap and with each dilution. Record the highest dilution which still produces symptoms on the inoculated plants.

C. Determination of Host Range

Grind one part infected plant tissue with five parts buffer, squeeze it through a cheesecloth, and inoculate various test plants (see section II A1).

D. Determination of Insect Vectors

When mechanical transmission fails, place insects on infected plants for acquisition feeding. After feeding, place the insects on healthy plants for transmission feeding, and observe the plants for symptom development.

E. Determination of Virus Identity by Serological Methods

Most serological methods are based on the precipitation produced when antibodies (the antiserum) and antigens (the virus) combine. Antisera must be prepared from purified or semi-purified virus preparations. Many antisera can also be ordered from:

ATCC (American Type Culture Collection)
12301 Parklawn Drive
Rockville
Maryland 20852
USA

The serological tests most commonly used are:

- Microprecipitation test in thin, small test tubes
- Ouchterlony agar gel double diffusion test in petri dishes
- Immunosorbent electron microscopy (ISEM)
- Enzyme-linked immunosorbent assay (ELISA)

These tests are applicable to virus identification from crude sap, clarified sap, and purified preparations. They require special training, and, in the case of ISEM and ELISA, special equipment which is not commonly available.

Ref: Milne, R.G., and E. Luisoni, E. 1977. Rapid immuno-electron microscopy of virus preparations. pp. 265-281. In: *Methods in Virology*, Vol. 6. K. Maramorosch and H. Kaprowski, eds. Academic Press, New York.

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Clark, M.F. and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34: 475-483.

IV. Storage of Viruses

- The most widely used storage method is to keep the virus in suitable, actively growing storage hosts.
- Virus infected plant material wrapped in plastic can be preserved in a freezer at -20°C .

Caution: infectivity may be lost through repeated freezing and thawing.

- Leaf material can also be rapidly dried and stored over calcium chloride at $0-4^{\circ}\text{C}$.

Materials needed:

- glass jar or petri dish
- granular anhydrous calcium chloride (5-15 mm)
- cotton, tissue paper, or gauze
- razor
- cellophane tape or parafilm

Method:

Place a few granules of anhydrous calcium chloride in the bottom of a glass jar or petri dish. Cover them with a thin layer of cotton, tissue paper, or gauze. On top of this layer place a 5 to 10 g sample of a virus-infected leaf which has been finely chopped with a clean razor blade. Cover the container, and seal it with tape or parafilm. In order to dry the leaf sample completely, it may be necessary to open the container several times and replace the calcium chloride crystals.

Ref: McKinney, H. H. and G. Silber. 1968. Methods of preservation and storage of plant viruses. In: K. Maramorosch and H. Koprowski (eds). *Methods in Virology*, Vol. 4. pp. 491-501. Academic Press, New York.

- Freeze drying

Special equipment is needed for this method.

Ref: Hollings, M. and O. M. Stone. 1970. The long term survival of some plant viruses preserved by lyophilization. *Ann. Appl. Biol.* 65, p. 411.

V. Control of Virus Diseases

Viruses so far cannot be controlled directly. Some antiviral compounds are known, but they are still in the developmental stages, and high costs and regulatory considerations have so far prevented them from being used on a large scale. Indirect controls remain the only practical method of controlling viruses. Here are a few of the methods most commonly used:

A. Preventing of Infection

- (1) Plant away from infected plants.
- (2) Crop rotation breaks the crop cycle and provides a host-free period. This method is particularly useful with viruses which have a limited host range.
- (3) Adjust planting time so as to avoid virus-carrying insect populations when the crop is young and plants are particularly susceptible to virus infection.
- (4) Control of insects that are virus vectors

- Use of insecticides

This gives good results for persistent aphid-, leafhopper-, and whitefly-transmitted viruses. It will not, however, control nonpersistent aphid transmitted viruses that have a short acquisition and inoculation feeding time. In these cases, the aphids will be able to transmit the virus before they are killed by the insecticide.

- Use of insect traps

- Color traps (yellow traps, also called Moericke pans, are useful for catching aphids and whiteflies).
- Light traps
- Suction traps
- Hormone (pheromone) traps

- (5) Elimination of weeds which are virus reservoirs and alternate hosts.

B. Minimizing the Spread of Virus Within a Crop

- (1) Eliminate virus-infected weeds or crops by roguing or herbicide application.
- (2) Minimize mechanical transmission by using clean tools for cutting and pruning (e.g. dipping the tools for one minute in a

saturated solution of trisodium phosphate (Na_3PO_4) at a pH of 11.7 or in skimmed milk minimizes transmission of TMV).

C. Using Healthy Planting Materials

Virus-free plants or plant parts can be obtained by:

(1) Special vegetative propagation

- Apical meristem culture (Meristematic tissue is generally free of virus.)
- Tip cuttings (Very young plant tips grow and elongate faster than the virus can occupy new tissue).

(2) Heat treatment

Heat treatments can inactivate or destroy the virus.

Hot water treatment

(Ratoon stunt of sugarcane, for example, can be controlled by immersing ratoons in 50°C water for 20 minutes.)

Hot air treatment

(Maintaining cassava mosaic-infected cuttings, for example, at 39°C for 28 to 42 days inactivates the virus.)

Ref: Nyland, G. and A. C. Goheen. 1969. Heat therapy of virus diseases of perennial plants. Annual Review of Phytopathology V. 7. p. 331-354.

Baker, K. F. 1962. Thermotherapy of planting materials. Phytopathology 52, 1244 - 1255.

(3) A combination of heat treatment and subsequent meristem culture.

Ref: Berg, L. A., and M. Bustamante. 1974. Heat treatment and meristem culture for the production of virus-free bananas. Phytopathology 64. 320-322.

(4) Clean seed

- Visual inspection

- Select seed from healthy looking plants.
- In cases where virus infection results in seed discoloration or abnormalities, select only healthy looking seed.

Ref: Phatak, H. C. 1974. Seed-borne plant viruses - Identification and diagnosis in seed health testing. Seed Science and Technology 2., pp. 3-155.

- Chemical treatments

Treatment of tomato seeds for 30 minutes with a solution of 12.5% trisodiumphosphate can eliminate TMV carried on the seed coat.

- Heat treatment

Exposure of seeds to high temperatures can sometimes eliminate viruses inside the embryo. It is important, however, for the seeds to first be brought to a low moisture content. Tomato seeds, for example, can be freed of internally carried TMV by being brought to a moisture content of approximately 4 to 8% and then heated to 78° dry heat for 2 to 3 days.

- Nucellar seedlings

This method is common in virus-free citrus propagation.

Ref: Reuther, W., Batchelor, L. D. and Webber, H. J. 1970. In: The Citrus Industry. V. 2: University of California, Division of Agricultural Science. 398 pp.

- (5) Resistant varieties that carry genes for resistance to the virus or the insect vector.

D. Cross Protection

This control method is based on the theory that a plant infected with one strain of a virus is often protected from infection by other related strains. Prior to the development of resistant varieties, it had been utilized in greenhouse tomato production to reduce yield losses due to tomato mosaic virus (TMV). When artificially inoculated at the seedling stage with experimentally produced, weak strains (attenuated strains) of TMV, tomato plants have been shown to be less severely damaged when subsequently infected with the naturally occurring strains of TMV.

Attenuated strains of viruses can be produced by treating naturally occurring strains with heat or with chemical mutagens such as nitrous acid.

VI. Useful References

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Plant Disease (Monthly, like Phytopathology, but covers aspects of applied plant pathology such as new diseases, epidemics, control, etc.)

Virology (monthly; includes all virus diseases, not only those of plants; concentrates on biochemical, biophysical aspects, serology, strain differentiation, protein and nucleic acid analyses.)