Biological diagnosis of virus and virus-like diseases: a special reference to stone fruit certification

Di Terlizzi B.

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Biological diagnosis oif virus and virus-like diseases: a special reference to stone fruit certification

Biagio DI TERLIZZI
Istituto Agronomico Mediterraneo
Valenzano, Bari (Italy)

SUMMARY - The biological diagnosis of virus and virus-like diseases of stone fruit trees was briefly discussed. Mechanical transmission and graft inoculation were separately exposed with a special reference to certification programs. Indexing facilities and the activities conducted in the framework of certification were also described. A list of virus and virus-like diseases of stone fruits and their main indicators were given. Finally, it was stressed that biological assays in general and woody indexing in particular still remain a compulsory tool for sanitary selection and certification programs.

Key words: stone fruits, plant viruses, virus-like diseases, biological diagnosis, plant certification.

RESUME - *Dans ce travail, on parcourt brièvement le diagnostic biologique des maladies à virus et de type viral des essences à noyaux. La transmission mécanique et l'inoculation par greffage sont discutées séparément, mais en se référant toujours aux programmes de certification. Les structures d'indexage et les activités conduites dans le cadre de la certification sont également décrites. En outre, une liste des maladies à virus et de type viral des essences à noyaux et de leurs indicateurs principaux est présentée. Enfin, on met en évidence que le diagnostic biologique en général et l'indexage sur des indicateurs ligneux, en particulier, demeurent encore une pratique indispensable pour la sélection sanitaire et les programmes de certification.*

Mots-clés: *essences à noyaux, virus des végétaux, maladies de type viral, diagnostic biologique, certification des plantes.*

I - Introduction

Diseases caused by virus or virus-like agents have induced heavy losses world-wide annually in fruit trees (Németh, 1986; Roistacher, 1992; Martelli, 1993). In fruit trees, more than 150 diseases caused by viruses, viroids, phytoplasmas, and unknown graft-transmissible, agents were reported. To control these diseases, it is important to identify the causal agent and to determine its properties. This has been possible in certain recalcitrant disease agents with recent advances in serological and molecular (probe hybridisation and PCR-based) techniques (Dunez *et al.*, 1994; Candresse, 1995). Nevertheless, biological indexing on herbaceous and woody plants remains as an essential tool for identifying and characterisation of new agents and diseases in certification programmes producing virus-free propagating materials. So long as the causal agent remains a mystery, the presence of a disease can be demonstrated only by biological transmissions to plant indicators.

II - Diagnosis problems for virus and virus-like agents in fruit trees

Some general considerations are needed when assaying samples for virus or virus-like agents in fruit trees: (i) the choice of plant tissues is important to achieve a successful diagnosis; (ii) also, the uneven distribution of infectious agents within the plant (i.e. plum pox virus, phytoplasmas) make appropriate tissue sampling critical; and (iii) environmental factors influence the agent concentration in a tree impairing success in virus detection when sampling in the different seasons of the year (e.g. virus concentrations usually higher in spring, and phytoplasmas and viroids in summer).

The large numbers of diseases reported for fruit trees were also due to the differential expression of the same pathogen(s) under different climatic conditions, plant species and varieties. Among the ilarviruses, *Prunus* necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) cause in the stone fruit trees 14 and 10 described diseases respectively (Dunez, 1988). Several diseases caused by apple chlorotic leaf spot virus (ACLSV) were also reported (Desvignes and Boyé, 1988). Moreover, diseased plants may be infected with more than one graft-transmissible agent making diagnosis more difficult.

When appraising the relative merits of different diagnostic methods, the following factors should be considered: (a) the sensitivity, i.e. the lower limits a virus may be measured or detected; (b) its accuracy and reproducibility; (c) the number of samples that can be processed in a given time; (d) the cost and sophistication of the apparatus and materials needed; (e) the level of training required for operators; and (f) adaptability to field conditions.

The last consideration may prove to be important in many Mediterranean countries and, the development of standard biological test procedures may be especially useful for these situations.

III - Biological indexing

Indexing may be defined as any test that reproducibly assess the presence or absence of a transmissible pathogen, or identify a disease on the basis of the reactions induced on specific indicator plants.

The recognition that symptoms caused by virus and virus-like disease could be reproduced from one plant to another by the transfer of a "contagium vivum fluidum" was significant for the birth of plant virology as a science. It was the end of the 19th century in the USA (Smith, 1888), when the infectious nature of peach yellows was demonstrated by graft transmission assays. Until the late 1950s, virus diagnosis was dominated by field symptomatology and biological tests. Early virologists learned that not only symptoms could be transferred from one plant to a similar one, but also that other genera of plants were susceptible infected and, moreover, that the symptoms produced on these plants were characteristic (diagnostic) for specific viruses. Although biological tests lack the clinical objectivity of other recently developed serological and molecular techniques, they still play a major role in routine diagnosis.

1. Mechanical inoculation to herbaceous plants

In detecting fruit tree viruses, mechanical transmission to herbaceous indicators was an important diagnostic procedure in further determining agent identity. The first mechanical transmission of a fruit tree virus was carried out successfully in the late 1940s, when cherry ringspot virus was transmitted to cucumber (Moore *et al.*, 1948). The use of some families of herbaceous plants has greatly improved the diagnosis of stone fruit viruses, e.g. *Cucurbitaceae* for ilarviruses, *Chenopodiaceae* and *Solanaceae* for nepoviruses and filamentous viruses. Beside virus diagnosis, herbaceous hosts were occasionally useful in strain identification. Šutic *et al.* (1971) identified three PPV strains on the basis of symptoms obtained on *Chenopodium foetidum* Schrad.

The value of herbaceous indicators reside in the fact they: (i) are easily to grow in greenhouse; (ii) develop symptoms rapidly and clearly; and (iii) allow rapid multiplication and build up of high virus titers for virus purification. Herbaceous plants are less costly compared to woody indicators, react more quickly when infected and allow for the detection of latent viruses which were not recognised otherwise. However, herbaceous indicators are limited because less than one-third of the known fruit tree viruses may be transmitted

mechanically. Not mechanical transmissibility of a virus may be not to the intrinsic properties of the virus itself, but to the source and acceptor plant (Németh, 1986).

Overall, herbaceous hosts, for certification purposes, are complementary to woody indexing and serological tests. The minimal host range of herbaceous host to be used in the sanitary controls for the certification of stone fruits should include: *Cucumis sativus* L., *Chenopodium quinoa* Wild., and *Nicotiana occidentalis* Weeler.

1.1. Inoculation procedure

The procedure for mechanical inoculation can be applied with a reasonable degree of success if some basic requirements in the management of herbaceous hosts and handling of the inoculum are met. In principle, parts of any living organ of an infected plant can serve as a source of inoculum for sap transmission. In practice, however, the chances for successful transmission are best if young, tender tissues from developing leaves, flowers or root tips were used. The collected samples should avoid exposure to direct sun and kept in a cold box during transportation. In the laboratory the samples should be processed immediately or may be stored in a refrigerator at 4°C for a short duration. Prolonged storage in a freezer at -20°C may be an option but not devoid of risk, for the particles of certain viruses may aggregate during the freeze process or disassemble during thawing, thus decreasing virus infective potential. For many viruses, the extraction medium is phosphate buffer, 0.1 M, pH 7 containing 2.5 % nicotine as antioxidant.

1.2. Symptom expression

Plants are grown and incubated after inoculation for symptom appearance at a temperature range between 18°C to 26°C. (Fig. 1-3).

2. Graft inoculation to woody indicators

As there are still many described *Prunus* diseases and viruses that can not be transmitted to herbaceous plants. With phytoplasma and xylem-limited bacteria, mechanical transmissions are practically impossible. Thus, remain the need for biological testing on woody indicators.

An ideal woody indicator plant must: (i) be free of viruses, while resistant to other non viral pathogens and pests; (ii) be easy to grow; (iii) react rapidly and specifically to a given virus; (iv) possibly be polyvalent, i.e. suitable for detecting more than one virus; (v) exhibit diagnostic symptoms under different conditions; and (vi) be available during the growing season. Since so few woody indicators posses so many attributes, research continually seek new, polyvalent indicators, surpassing in quality previous indicators.

Woody indicators may be either cultivated varieties susceptible to individual viruses or wild-growing species propagated on virus-free rootstocks. In the latter case, the selection of suitable rootstocks is very important. The sweet cherry (*Prunus avium* L.) cv. Bing, produce more enations when on *P. mahaleb* rootstock than on *P. avium;* and *P. serrulata* cv. Kwanzan grows better on *P. avium* than on *P. mahaleb* L. (Németh, 1986). In many cases, the rootstock itself can be used as an indicator plant i.e. *P. persica* (L.) Batsch cv. GF 305, *P. tomentosa* Thunb., *P. mahaleb* or the indicator can be self-rooted *in vitro* (Shirofugen, GF 305).

2.1. Field indexing

The method involve T-bud or chip-bud grafts onto an indicator tree grown in the field. Grafted trees must be observed for, at least, two growing seasons and longer when indexing for fruit marker diseases. Field indexing is indispensable for the reliable detection of virus-like diseases of stone fruits.

In different indexing centres, woody indexing may be initiated in greenhouse for 4-5 months at 22-24°C. Under greenhouse conditions, plants are checked weekly for symptoms and used to detect cherry green ring mottle on *P. serrulata* cv. Shirofugen; the ilarviruses, nepoviruses, PPV, and ACLSV on GF 305; cherry twisted leaf, and cherry mottle leaf on *P. avium* cv. Bing and apricot pit pox on *P. armeniaca* cv. Tilton. After the preliminary observations, indicator plants must be transplant in the field and observed for one or more seasons to detect other diseases (i.e. *P. avium* cvs. Bing or Sam for cherry rusty mottle, cherry necrotic rusty mottle, stem pitting; and *P. armeniaca* cv. Luizet for apricot leaf roll).

2.2. Glasshouse indexing

Although woody indexing of fruit tree virus and virus-like diseases is preferred, field indexing may prove too costly in terms of time, space and labour. So, efforts have been made to transfer indexing from the field to the glasshouse, utilising new indicators and to improve the detection of several agents (Fridlund,1970; Desvignes, 1976; Boyé and Desvignes, 1986; Cornaggia and Desvignes, 1986; Gilles and Bormans, 1986). The change from field to greenhouse indexing became possible because the appropriate management of environment and hosts yield more rapid and accurate results. The average time for symptom development may be reduced to a few months in greenhouse, compared to one to five years in the field (Fridlund, 1980a,b).

Diagnostic symptoms developed in most host-virus combinations occurred at 18 or 22°C. However, the optimum temperature for maximum symptom intensity of a particular disease was shown to vary substantially among different indicators (Fridlund, 1970).

More recent studies suggest that greenhouse indexing can substitute for field indexing for several virus diseases. However, until a complete correlation between greenhouse results and field observations is made, field indexing must continue (Fridlund, 1980a)

Greenhouse indexing has had a sudden boost with the use of the polyvalent GF 305 peach indicator for the indexing of graft-transmissible agents of stone fruit trees (Bernhard and Marenaud, 1962; Bernhard *et al.*, 1969). Depending on the virus, indexing on GF305 may require as little as 3 weeks to some months. By this method ilarviruses, nepoviruses, PPV, and ACLSV, as well as some phytoplasmas and virus-like diseases may be detected. The time needed for symptom expression was related to how the indicator was utilised (self-rooted or grafted on a rootstock). When simple indexing was combined with the cross-protection technique, peach latent mosaic was readily detected (Desvignes, 1976; Boyé and Desvignes, 1996).

IV - Indexing facilities for a certification program

The use of differential woody indicators is compulsory in any certification program because all diseases irrespective of causal agents, are detected by graft-grafting.

Indexing programmes require adequate facilities in terms of: (i) greenhouse for biological tests; (ii) indicator mother plots and fields for bioassays; (iii) grafting area; (iv) sheds and rooting benches; (v) forcing chambers; (vi) cold chambers; (vii) soil mix box; (viii) storehouse; (ix) screenhouse, and (x) diagnostic laboratory.

Availability of nursery land is of utmost importance for growing mother tree indicators and indicators that have been graft-inoculated.

1. Indexing

1.1. Establishment and care of mother indicator plants

Mother indicator plantings constitute the source of wood or seeds for indexing. Errors in the establishment and care of these plantings may compromise indexing programmes.

Mother plant plots producing stone fruit indicators must be: (i) located on grounds reasonably close to the research unit in charge of indexing; (ii) established on good quality, well-drained, and clean soil, preferably with no fruit tree history or at least free of them for at least 5 years; (iii) at least 100 m far from other fruit orchards in case of mother plants for bud production and 300 m for plants used in seed production (*P. mahaleb*, GF305); (iv) in place for no more than 10 years if destined for budwood production and 12 years for seed production; (v) not allowed to bloom if destined to bud production; (vi) protected from possible contamination from adjacent plots through irrigation, water, flooding and cultivation; and

(vii) large enough to accommodate other optional indicators in addition to those used routinely.

The soil must be free from nematodes, in particular the virus vector species. Spraying schedules for the chemical control of airborne vectors should be devised according to necessity and local conditions.

1.2. Care of grafted plants

Under greenhouse, screenhouse, or open field cultivation, indicators must be protected from foliar diseases and pests, that may obscure symptoms and even endanger their survival. For field-grown indicators, cultural practices were similar to those routinely used in nurseries.

Field readings are done four times a year, two in between March to May, once in summer and again in autumn. Careful records of readings must be maintained. The time and appearance of symptoms, type and severity are noted and compared with the responses of positive controls. Each plot should also contain negative controls, i.e. healthy, non-inoculated indicator plants. The positive and negative controls should be distributed in 2-3 replications over the indexing plot.

2. Graft-inoculations

The various types of grafts used in the indexing process are those used in horticultural practice. The choice depend on : (i) the development stage of scion and rootstock; (ii) the season grafts are done; and (iii) the quantity of available plant material. Grafting techniques used are illustrated in several bibliographies (Németh, 1986; Desvignes, 1990; Roistacher, 1992; Martelli, 1993).

V - Stone fruit indicators

A list of *Prunus* viruses and viroids mentioned in the text is given in Tab. 1, and *Prunus* indicator plants are listed in Tab. 2. Even through the indicators are susceptible to a whole range of graft-transmissible disease agents, some of them develop specific diagnostic symptoms for a given virus or diseases agent.

1. Symptoms of diseases and agents

A comprehensive description of the subject has been reported in several books and papers (Németh, 1986; Desvignes, 1990; Boyé and Desvignes, 1996; Barba *et al.*, 1998).

(i) Ilarviruses (PDV, PNRSV, ApMV) generally induce, on *P. persica* GF 305 or Elberta in greenhouse conditions, delayed budbreak, reduced shoot growth, and chlorotic line pattern (mainly for ApMV) (Fig. 5); on *P. serrulata* cv. Shirofugen infected inoculated

buds cause local necrosis when infected with PNRSV or PDV; on *P. avium* cv. Bing, in open field PDV cause chlorotic rings and spots (Fig. 4).

(ii) Nepoviruses (ArMV, TomRSV, TBRV, SLRV, RRSV, CLRV, CRLV, MLRSV) generally induce, in *P. persica* GF 305 or Elberta under greenhouse conditions, reduced growth, stunting, rosetting (Fig. 6). Symptoms of TomRSV may develop after a second season of growth.

(iii) ACLSV: cause on GF 305 dark-green small spots, slight deformation of the leaf surface and, for some strains, severe spotting and leaf deformation.

(iv) PPV: cause on *P. persica* GF 305 or Elberta under greenhouse conditions, vein clearing and distortion of young leaves (Fig. 7). In the field, *P. tomentosa* show distortion and epinasty of first leaves; later chlorotic spots which turn necrotic by mid-summer.

(v) Viroids: peach latent mosaic viroid (PLMVd) was detected successfully on GF 305 by cross-protection with a severe strain of it (Fig. 8). In contrast, hop stunt viroid (HSVd) indexing on GF305 was not very reliable.

(vi) Phytoplasmas: apricot chlorotic leaf roll under field conditions induce leaf rolling on *P. armeniaca* cvs. Luizet or Priana, On GF305 and under greenhouse conditions leaves become chlorotic, turn reddish and abscise after 3-4 months. The most severe infected plants wilt rapidly. Other phytoplasmas (cherry X disease, peach yellows, peach rosette, etc) were detected on GF305.

(vii) Virus-like diseases: There are several graft-transmissible diseases of stone fruits whose causal agents have not been determined. These diseases must use woody indexing for their detection. A list of these diseases and woody indicators was included in Tab. 2.

In Tab. 3, an indexing protocol in a certification program for stone fruits was outlined.

VI - Conclusions

Despite disadvantages of being laborious, time-consuming, and skill-demanding, woody indexing remains a compulsory approach, in sanitary selection and certification of propagative materials. In the future, even with the advances in new technologies for improved diagnostic reagents and methods, the biological indexing remains the bases of a certification programme.

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Tab. 1. Viruses and viroids: their acronyms and taxonomic group

English name	Acronym	Taxonomic group
Virus		
Apple chlorotic leaf spot	ACLSV	*Trichovirus*
Apple mosaic	ApMV	*Ilarvirus*
Arabis mosaic	ArMV	*Nepovirus*
Cherry green ring mottle	CGRMV	*Foveavirus*
Cherry leaf roll	CLRV	*Nepovirus*
Cherry rasp leaf	CRLV	*Nepovirus*
Little cherry	LChV	*Closterovirus*
Myrobalan latent ringspot	MLRSV	*Nepovirus*
Plum pox	PPV	*Potyvirus*
Prune dwarf	PDV	*Ilarvirus*
Prunus necrotic ringspot	PNRSV	*Ilarvirus*
Raspberry ringspot	RRSV	*Nepovirus*
Strawberry latent ringspot	SLRV	*Nepovirus*
Tomato black ring	TBRV	*Nepovirus*
Tomato ring spot	TomRSV	*Nepovirus*
Viroid		
Hop stunt	HSVd	
Peach latent mosaic	PLMVd	

***Tab.* 2.** Principal indicators for the detection of virus and virus-like diseases in the stone fruit certification programs

Indicators	Viruses and virus-like diseases detected
Prunus persica cv. GF305 or Elberta	ACLSV, ApMV, PPV, PDV, PNRSV, CLRV, CRLV, ArMV, RRSV, SLRV, TBRV, TomRSV, MLRSV, PLMVd, apricot chlorotic leaf roll
P. persica cv. Springtime	peach asteroid spot
P. armeniaca cv. Luizet or Priana	apricot chlorotic leafroll
P. armeniaca cv. Luizet or Tilton	apricot ring pox
P. avium cv. Sam	LCV, cherry necrotic rusty mottle, cherry European rusty mottle
P. avium cv. Bing	CMLV, cherry rusty mottle, cherry twisted leaf, cherry European rasp leaf, nepoviruses
P. avium cv. Canindex	LChV
P. serrulata cv. Shirofugen or Kwanzan	CGRMV, PNRSV, PDV
P. tomentosa IR 473/1 or IR 474/1	PNRSV, PDV, ACLSV, TomRSV, PPV, apricot ring pox

Tab. 3. Indicated protocols to assess the sanitary status of propagating material for the production of "virus-free" category of stone fruits

Agent	Species	Woody indicator	Herbaceous indicator (*)	Other tests
Viruses				
ACLSV	(1,2,3,4,5)	GF305	(*)	ELISA
ApMV	(1,2,3,4,5)	GF305	(*)	ELISA
ArMV	(3)	GF305	(*)	ELISA
CGRMV	(3,4)	Kwanzan or Shirofugen	-	RT-PCR
CLRV	(3)	GF305	(*)	ELISA
CRLV	(3)	GF305	-	ELISA
MLRSV	(5)	GF305	(*)	ELISA
PDV	(1,2,3,4,5)	GF305	(*)	ELISA
PNRSV	(1,2,3,4,5)	GF305	(*)	ELISA
PPV	(1,2,3,4,5)	GF305	(*)	ELISA
RRSV	(3)	GF305	(*)	ELISA
SLRV	(3,4)	GF305	(*)	ELISA
TBRV	(3,4)	GF305	(*)	ELISA
TomRSV	(3,4,5)	GF305	(*)	ELISA
CMLV	(3)	Bing	-	ELISA
LChV	(3)	Sam	-	RT-PCR
Viroid				
PLMVd	(4)	GF305	-	RT-PCR
Phytoplasma				
Apricot chlorotic leafroll	(2,4,5)	Luizet or Priana	-	PCR
Virus-like diseases				
Apricot ring pox	(2)	Luizet or Tilton	-	-
Cherry twisted leaf	(3)	Bing	-	-
Necrotic rusty mottle	(3)	Sam	-	-
Peach asteroid spot	(2,4)	Springtime	-	-
Rusty mottle(European)	(3)	Sam or Bing	-	-

(*) The herbaceous indicators for mechanically transmissible viruses are: *Chenopodium quinoa, Cucumis sativus* cv. Marketer and *Nicotiana occidentalis.*

(1) almond; (2) apricot; (3) cherry; (4) peach; (5) plum.

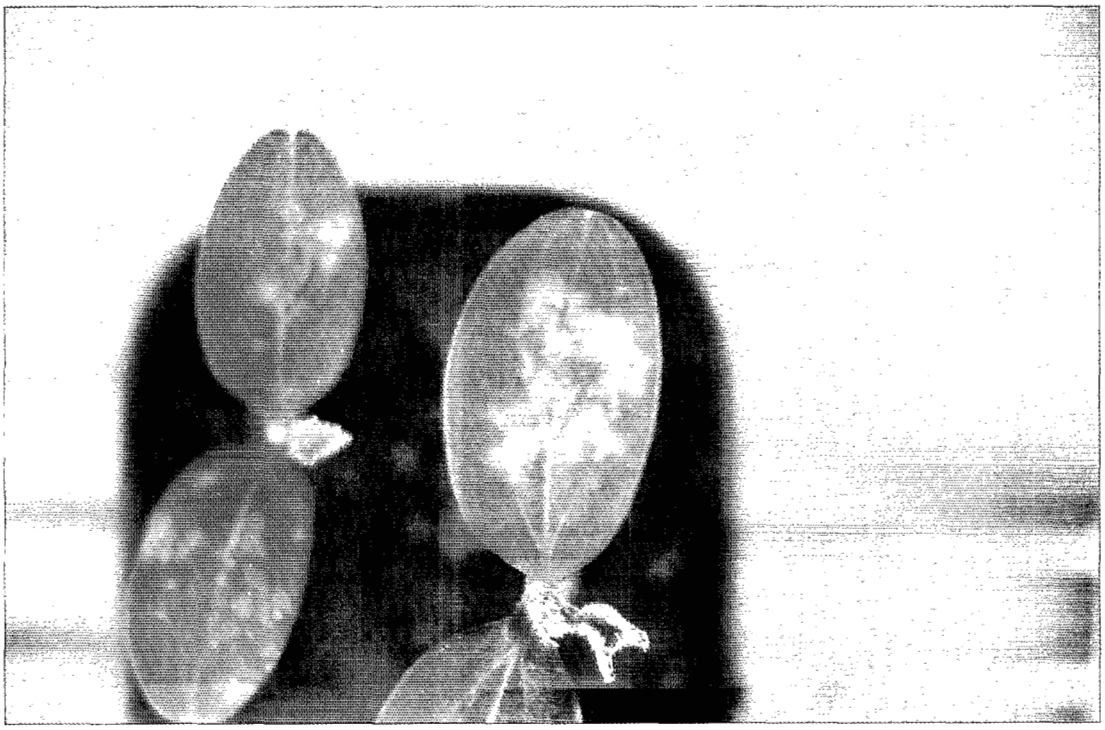

Fig. 1. Chlorotic local lesions on cucumber cv. Marketer caused by PNRSV

Fig. 2. Yellow mosaic on cucumber cv. Marketer caused by ApMV

Fig. 3. Chlorotic spots on *C. quinoa* associated with the presence of ACLSV

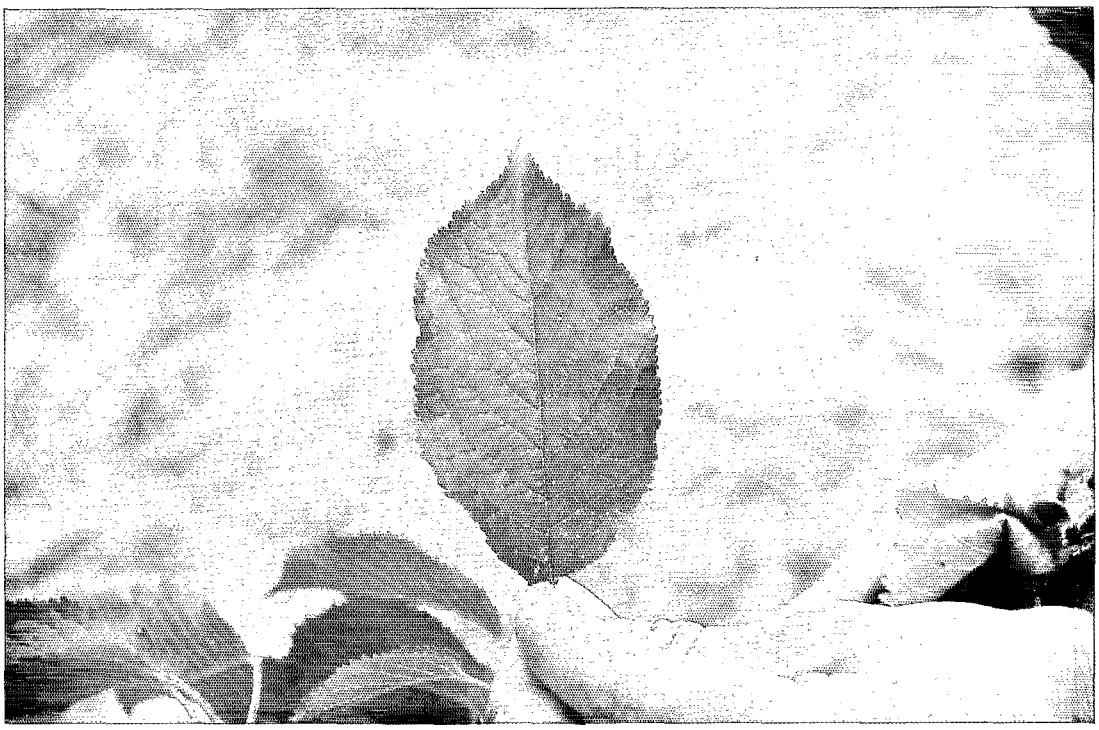

Fig. 4. Chlorotic pattern and rings on *P. avium* cv. Bing caused by PDV

Fig. 5. Yellow pattern on GF305 caused by ApMV

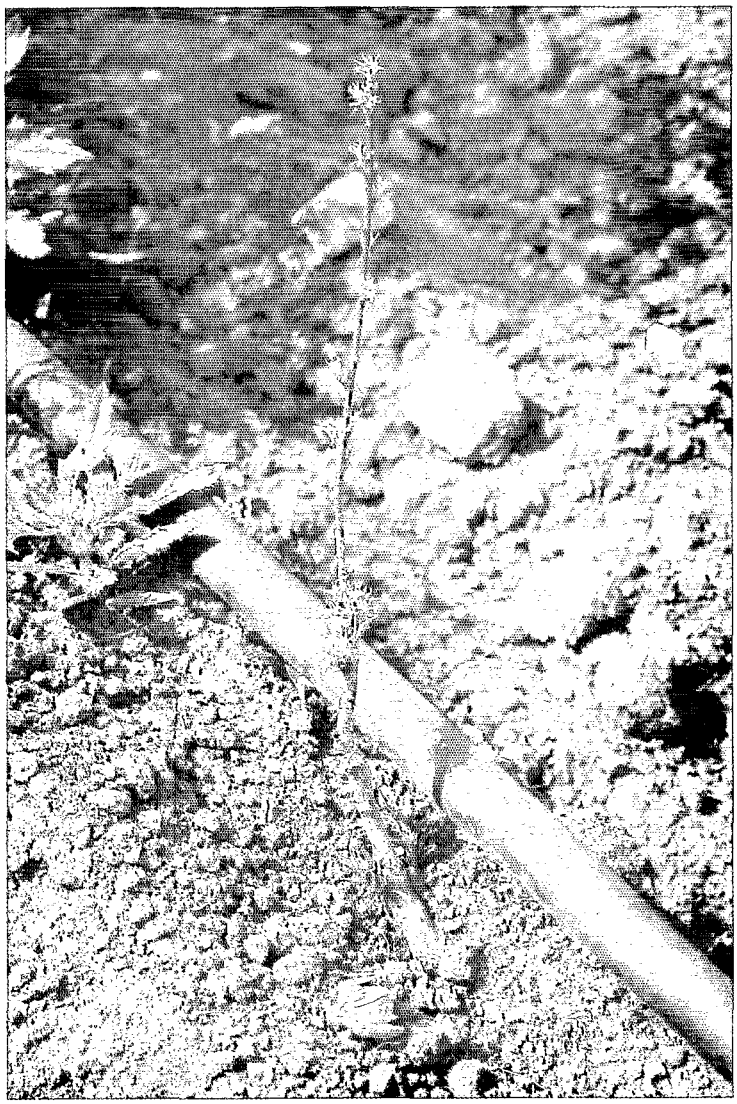

Fig. 6. Stunting and rosette of GF305 infected with CLRV

Fig. 7. Chlorotic areas along the vein and leaf deformation caused by PPV on GF 305

Fig. 8. Chlorotic spots and areas in the leaves of GF305 caused by PLMVd