Vitis 18, 127—136 (1979)

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Identification of virus diseases of grapevine and production of disease-free plants[1])

by

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Der Nachweis von Rebvirosen und die Erzeugung virusfreien Pflanzgutes

Zusammenfassung. — In dem vorliegenden Übersichtsbericht werden symptomatologische Probleme, der Virusnachweis mit Hilfe von krautigen Testpflanzen und Indikatorreben, serologische Nachweismethoden sowie die Möglichkeiten elektronenmikroskopischer Verfahren behandelt. Abschließend werden die bislang praktizierten Programme für die Erzeugung virusfreien Vermehrungsmaterials diskutiert.

Symptomatology

Symptomatological responses of grapevine (*Vitis vinifera* L.) to virus infections are only partially diversified and specific. The boundary of many virus-induced disorders is often difficult to establish because of the limited distinctiveness of symptoms displayed in the field by affected plants. Thus, some of the entries of Table 1, rather than diseases in their own right, are to be considered simply as more or less complex syndromes which may be induced by more than a viral pathogen. For instance, both sap-transmissible (i.e. grapevine fanleaf [GFV], tomato ringspot [TomRSV] and tobacco ringspot [TRSV]) and non sap-transmissible disease agents have been found associated with disorders like enations and legno riccio (rugose wood, stem pitting), but their true incitants have not yet been identified (see recent reviews by Goheen [1977] and Martelli [1978]). Likewise, leafroll, which seems induced by a potyvirus in Israel (Tanne *et al.* 1977), may not have the same causal agent elsewhere, should failures to isolate that virus in other countries be taken as indications that it does not occur there.

Sap-transmissible viruses (Table 2) pose even greater problems. In fact, mottling and various degrees of leaf deformity are likely to be induced by any of the 11 nepoviruses found so far in grapevine or by tobacco necrosis and Joannes-Seyve virus (Cesati and van Regenmortel 1969, Dias 1973). Similarly, different patterns of chrome-yellow discolouration ranging from extensive yellowing of the leaves to occasional yellow spots or mild vein banding can be elicited by any of the following pathogens: GFV, TomRSV, arabis mosaic (AMV), grapevine chrome mosaic (GCMV), alfalfa mosaic (LMV) and grapevine yellow speckle (GYSV). Asteroid mosaic and fanleaf diseases are also hardly distinguishable in nature.

[1]) The present review is largely based on an invitation paper given at the International Symposium on Virus Diseases and Bacterial Canker of Grapevine, Pleven, Bulgaria, 29—30 May, 1978.

Publication No. 28 of the "Progetto Finalizzato C.N.R. Miglioramento delle produzioni vegetali per fini alimentari ed industriali mediante interventi genetici. Sottoprogetto Vite ad uva da vino".

It is then evident that in none of the above cases a reliable diagnosis can be made on the basis of field symptomatology alone. Identification must be carried out with laboratory tests not only in these instances but also with diseases like corky bark, fleck, vein mosaic, vein necrosis and yellow speckle, whose agents are semi-latent or latent in most European grape cultivars.

Indexing procedures

A. Herbaceous indexing

Mechanical transmissions are performed by crushing in a mortar leaves of candidate vines in presence of buffered solutions. Young leaves with symptoms, either from field-grown plants or from glasshouse-forced cuttings make an adequate inoculum source. Alternatively, young succulent root tips from sand-forced cuttings can be utilized. In the latter case, tissues are crushed in conventional Na-K) phosphate buffer 0.1 M, pH 7—7.2, whereas with leaves, especially if they are aged and coming from vines grown outdoors, extraction in a 2—3 % aqueous nicotine solution is recommended (CADMAN *et al.* 1960). Nicotine-phosphate buffers (UYEMOTO 1975) or additives like DIECA, bentonite, activated charcoal etc., may increase the number of positive transmissions. Good results seem to be yielded also by a procedure recently developed (JANKULOVA 1978 and personal communication) in which grape leaves are crushed in 4 vol of 0.1 M phosphate buffer containing 5 % polyvinylpyrrolidone and 4 % polyethylene glycol 6000. The slurry is centrifuged at low speed, the pellets are resuspended in distilled water, stored with the supernatant at −20 °C for about 4 weeks, then thawed and used as inoculum.

Phenol extraction of infected grape leaves, which has proven essential for isolating the virus associated with leafroll symptoms in Israel (TANNE *et al.* 1974, 1977), is worth trying with viruses that have so far resisted mechanical transmission.

Initial inoculations are customarily performed on a limited range of herbaceous hosts comprising 2—3 actively growing plants of *Chenopodium quinoa* WILLD. (8-leaf stage), *C. amaranticolor* COSTE et REYN. (8-leaf stage), *Phaseolus vulgaris* L. (primary leaves half expanded), *Gomphrena globosa* L. (6-leaf stage), *Cucumis sativus* L. (cotyledonary stage), *Nicotiana clevelandii* GRAY and/or *N. benthamiana* DOMIN. (4 to 6-leaf stage). The majority of grapevine viruses can be isolated on these hosts with the first inoculation attempt and can subsequently be transferred to a wider host range for tentative identification.

Differential host reactions are not always useful for distinguishing different viruses and can be misleading unless they are highly characteristic. For example, nepoviruses can hardly be distinguished from one another on the basis of symptoms induced in herbaceous hosts. Even the peculiar twisting of the top leaves of *G. globosa*, so typical of GFV infections (HEWITT *et al.* 1962), is not specific since it can be evoked also by ordinary AMV grape isolates (VUITTENEZ *et al.* 1968).

Host range responses are therefore very useful for sorting out viruses that occur in mixed infections and for a preliminary approach to their identification, but often require support by more refined and reliable methods like serology and electron microscopy.

Herbaceous indexing gives rapid results as symptoms may develop within 1—2 weeks from inoculation. However, it can be performed satisfactorily only in spring, when virus concentration in the leaves is relatively high. With increasing temperatures the method becomes less dependable, unless the source material derives from glasshouse-grown cuttings.

Table 1

Virus diseases of grapevine, their geographical distribution, diagnostic and therapeutic methods
Viruskrankheiten der Rebe, ihre geographische Verbreitung sowie Diagnose- und Therapiemethoden

Disease	Geographical distribution	Mechanical transmissibility of agents	Diagnostic methods[1])	Woody indicators	Duration of heat treatment (d)
1. Infectious degeneration complex					
a. Fanleaf and related diseases	Worldwide	Yes	S, HI, WI	*V. rupestris*	30—35
b. Diseases induced by other European NEPO viruses	Europe	Yes	S, HI, WI	*V. rupestris*	30—35 (AMV, TBRV, CGMV)
2. Grape decline (American NEPO viruses)	U.S.A., Canada	Yes	S, HI, WI	Several French hybrids and *V. vinifera* cvs.	50 (TomRSV)
3. Enations	Probably worldwide	Uncertain	WI	Italia, Kober 5 BB	Unknown
4. Legno riccio (stem pitting, stem grooving)	Worldwide	Uncertain	WI	420 A, Kober 5 BB, *V. rupestris*	Unknown
5. Leafroll	Worldwide	Uncertain	WI	LN-33, Baco 22 A, Pinot noir Cabernet franc, Mission, Prokupack	60—120
6. Corky bark	Probably worldwide	No	WI	LN-33	More than 90
7. Fleck	Probably worldwide	No	WI	*V. rupestris*	80—120
8. Vein mosaic	Several European countries Australia (?)	No	WI	*V. riparia* Gloire de Montpellier	Unknown
9. Vein necrosis	Several European countries	No	WI	110 R.	Unknown
10. Yellow speckle	Probably worldwide	No	WI	Mission seedling 1, Esparte Rcatzitelli (?)	Heat resistant
11. Asteroid mosaic	U.S.A. (California)	No	WI	*V. rupestris*	40—45

[1]) S = Serology; HI = Herbaceous indicators; WI = Woody indicators.

B. Woody indexing

This method represents by far the most widespread technique for identifying virus of grapevine. It is based upon the differential reactions that some *Vitis* species and their hybrids express when artificially infected (Table 1). The symptoms are often specific enough for a reliable diagnosis: e.g. 110 R. for vein necrosis, *V. rupestris*, for fleck or asteroid mosaic, red-berried *V. vinifera* cultivars for leafroll, LN-33 for corky bark, rootstock hybrids for legno riccio (see for details MARTELLI 1975, GOHEEN 1977, UYEMOTO *et al.* 1978). Woody indexing can be performed in several ways:

Table 2

Mechanically transmissible viruses isolated from *Vitis*, their vectors and geographical distribution

Aus *Vitis* isolierte mechanisch übertragbare Viren, ihre Vektoren und die geographische Verbreitung

Virus	Vector	Geographical distribution
1. Grapevine fanleaf[1])		
Distorting strains	*X. index*	Worldwide
	X. italiae	Worldwide
Yellow mosaic strain	*X. index*	Worldwide
Vein banding strain	*X. index*	Worldwide
2. Arabis mosaic[1])		
Type strain	*X. diversicaudatum*	France, Germany, Hungary, Bulgaria, Switzerland
3. Tomato blackring[1])		
English strain	*L. attenuatus*	Germany
4. Raspberry ringspot[1])		
Bercks' isolate	Unknown	Germany
Palatinate strain	Unknown	Germany
5. Strawberry latent ringspot[1])		
Type strain	Unknown	Germany
6. Grapevine chrome mosaic[1])	Unknown	Hungary, Czechoslovakia
7. Artichoke Italian latent[1])	Unknown	Bulgaria
8. Peach rosette mosaic[1])	*X. americanum*	U.S.A. (Michigan), Canada
9. Tomato ringspot[1])		
Yellow vein strain	*X. americanum*	U.S.A. (California)
Type strain	Unknown	U.S.A. (New York), Canada Yugoslavia (?)
10. Tobacco ringspot[1])	Unknown	U.S.A. (New York)
11. Grapevine Bulgarian latent[1])		
Type strain	Unknown	Bulgaria, Portugal
New York strain	Unknown	U.S.A. (New York)
12. Tomato bushy stunt	Unknown	Germany, Italy, Czechoslovakia
13. Alfalfa mosaic	Unknown	Germany, Switzerland Czechoslovakia, Bulgaria
14. Tobacco mosaic	Unknown	Germany, Bulgaria, Italy, U.S.A., Yugoslavia, U.S.S.R.
15. Joannes-Seyve virus	Unknown	Canada
16. Cv. Elbling virus	Unknown	Germany
17. Sowbane mosaic	Unknown	Germany
18. Tobacco necrosis	Unknown	South Africa
19. Bratislavia mosaic	Unknown	Czechoslovakia
20. Potato X	Unknown	Italy
21. Broad bean wilt	Unknown	Bulgaria
22. Leafroll (Israel)	Unknown	Israel

[1]) NEPO viruses.

1. Green grafting. This method has been developed in Australia (TAYLOR *et al.* 1967) and it is used successfully in other countries as well (HEVIN *et al.* 1973). Essentially it consists in grafting a piece of a green shoot, 3—4 mm in diameter, on an actively growing green stock. The graft is sealed with latex bandage and is protected from dessication by a plastic wrapping at the bud union. The indicator can be used as scion or stock and grafting can be done outdoors (under mild climatic conditions) or in a climatized glasshouse.

The main advantage of green grafting is the rapidity of the indicator's reaction, i.e. 2—3 weeks for fanleaf and related diseases, 8—10 weeks for leafroll (TAYLOR *et al.* 1967, HEVIN *et al.* 1973).

2. Chip-budding is a highly dependable procedure developed in California (HEWITT *et al.* 1962) in which one or more chip-buds, taken from cold-stored dormant wood of the candidate vine, are inserted just below the terminal shoot of an indicator cutting in full growth (for details see UYEMOTO *et al.* 1978). A high percentage of successful graft unions is obtained but symptoms expression may be rather slow. Usually, shock symptoms induced by nepoviruses appear 30—50 days after budding, whereas clearcut responses to corky bark and leafroll may take as long as 16—18 months.

Recently, the method has been modified so as to shorten considerably the incubation period for symptoms detection. This is achieved by placing chip-bud grafted LN-33 cutting in a growth chamber with controlled temperature and illumination (MINK and PARSONS 1975, 1977). The indicators are kept under continuous illumination (10,000 lx) but are subjected to alterate cycles of temperature regimes (22 °C for 3 weeks, then 32 °C for 3 weeks, then again 22 °C) to induce early symptomatological responses. In this way, fanleaf, leafroll, corky bark and yellow speckle can be indexed within a 2-month period (MINK and PARSONS 1977).

3. Whip-grafting is the ordinary bench-grafting technique in which dormant cuttings of the candidate vine are used as rootstocks and buds of the indicator as scions. Grafts are forced in sawdust and are planted out in a nursery. In our laboratory, for each clone to be indexed a total of 25 whip-grafts are made with the following indicators: *V. rupestris*, LN-33, *V. vinifera* cv. Mission, 110 R., 420 A or Kober 5 BB.

With this method several thousand grafts can be made in a few days, but graft take is not always satisfactory and symptom expression must be evluated over a 2-year period.

Whatever the method used, indicator vines are usually transplanted outdoors some time after grafting. Readings for symptoms are made twice a year. With certain diseases (e.g. vein mosaic), however, better symptomatological responses are obtained under glasshouse conditions.

Serology

Serology is a most valuable method, fast, reliable and specific but, with grapevine viruses, is biased by: a) lack of availability of antisera other than to sap-transmissible viruses; b) exceedingly low concentration of viral antigens in grape leaves for detection in expressed sap with ordinary techniques.

Only tobacco necrosis and grapevine Bulgarian latent viruses are reported to occur in sufficiently high concentration in naturally infected grapevines to produce clear-cut serological reactions in gel double diffusion tests (CESATI and VAN REGENMORTEL 1969, MARTELLI *et al.* 1977).

VUITTENEZ and coworkers have partially overcome the problem of low antigen concentration by extracting sap from grape leaves of the spring growth and pelleting viruses by ultracentrifugation (for details see VUITTENEZ 1970). These extracts contain enough viral antigens to yield visible precipitin lines in gel diffusion tests. This procedure is time consuming and not full proof, and, although it is more reliable when applied to extracts from herbaceous hosts infected with viruses previously transmitted from diseased vines, still is rather unpractical and requires adequate laboratory equipment.

The antibody-sensitized latex test is far more practical, and, owing to its sensitivity, which is 25—100 times higher than tube-precipitin (ABU SAHLI *et al.* 1968) and dependability (BERCKS [1973] scored positive reactions in 86 out of 100 infected vines), is gaining increasing favour.

The latex test has been adapted to plant virus diagnosis by BERCKS and coworkers (for details see BERCKS 1967, BERCKS and QUERFURTH 1969) and now finds practical application in grapevine indexing. In Germany, for example, about 8,000 vines were subjected to serological screening for 6 sap-transmissible viruses in 1977 (H. L. PAUL, personal communication).

More recently, another serological test based on the use of enzyme-labelled antibodies has been developed for detecting plant viruses in crude extracts (for details see VOLLER *et al.* 1976, CLARK and ADAMS 1977). This technique, known as ELISA (enzyme-linked immunosorbent assay), is highly specific, reliable and more sensitive than the latex test. ELISA applications to plant extracts from fruit trees infected by both elongated and isometric viruses have proven so satisfactory, that the method should be investigated in detail also for grapevine virus identification. Preliminary results indicate that ELISA has been used satisfactorily for the detection of leafroll in Germany (R. CASPER, personal communication) and GFV in Switzerland (R. BOVEY, personal communication) and Italy (unpublished information).

Electron microscopy

Low virus concentration in naturally infected grapevine leaves not only impairs direct serological identification, but also prevents the use of electron microscopy as a rapid detection means. In fact, chances of observing virus particles in dip preparations from grape material are remote, even if strong symptoms are shown by the mother plant. On the other hand, in very few instances virions have been visualized in thin sections of grapevine tissues (RUSSO 1975). It appears, therefore, that electron microscopy as such may not be very helpful, even for preliminary screenings.

However, it does not seem that the potentialities of relatively recent methods such as immunoelectron microscopy, have been explored with reference to grapevine viruses. Immunoelectron microscopy is a quick and highly specific technique which, as reviewed by MILNE and LUISONI (1978), consists in procedures aiming both at increasing the number of virus particles from leafdips that remain on the microscope grid, so that they do not escape observation (e.g. clumping, DERRICK's antibody adsorption), and at identifying the viruses trapped on the grid by coating them with specific antibodies (decoration).

Production of virus disease-free plants

The indiscriminate exchange and marketing of uncertified graftwood and rootstocks can be regarded as the single cause that has contributed the most to the dissemination of destructive virus diseases throughout the world. As a consequence, except for a few fortunate enclaves like South Australia, where no new grapevine introductions have been made since the late 1800s (Francki and Crowley 1967), the sanitary conditions of the plantings in many leading viticultural areas of the world have steadily and dramatically detertiorated in the last 50—60 years.

Eventually, the situation has become so bad that several countries have felt the compelling need for the estabilshment of some kind of sanitation programme for producing "clean" (virus disease-free) clonal stocks (for a review see Bovey *et al.* 1975).

A primary example of a successful certification programme is that of California, where almost 65 million certified plants have been produced since 1960 and 95 % of all grapes sold in 1976 were of certified stocks (Goheen 1977).

Virus disease-free plants can be obtained through short or long term programmes. The former are essentially based on visual selection carried out by choosing vigorous, productive, apparently healthy plants, and propagating from them. Indexing may or may not accompany selection work, but it should be always recommended.

Under certain favourable conditions (Bovey *et al.* 1967), this procedure permits a rapid improvement of sanitary standards, through the elimination of severe forms of major virus diseases, by carefully observing candidate vines when symptom expression is at its best: 1) in spring, for leaf and cane deformation, wood pitting and chromatic disorders of the foliage (chlorotic mottles, various types of yellow discolourations, etc.); 2) in autumn, for cluster abnormalities, reddening on the leaves and, after leaf shedding, cane deformation.

Of course, infections by mild strains of main pathogens are likely to escape attention and latent viruses cannot be detected. Hence visual selection is not be relied upon as the only means for a real and long lasting sanitary progress of viticulture.

Long term programmes can be envisaged as interdisciplinary enterprises requiring contribution of different competences. They are based on clonal and sanitary selection, production of disease-free material by heat treatment and technological evaluation of certified clones.

One such programme for the improvement of wine grape cultivars is now under way in Italy, sponsored and financed by the Italian National Research Council. The cooperating parties are 22 research units composed of viticulturists, virologists and enologists who operate in the major viticultural districts of the country. Over 4000 "presuntive" clones of more than 200 different wine grape cultivars are presently under evaluation (Scaramuzzi 1977). Procedures for registration of clones conform to those established by the Economic European Community (CEE). The estimated time for registration of "new" certified clones, including heat therapy and indexing, ranges from 8 to 10 years, which is about half the time required in other countries (e.g. Germany) by similar programmes.

There is no doubt that heat therapy is a necessary complementation of clonal and sanitary selection. Different procedures may be followed, all aiming at eliminating obnoxious viruses from shoot tips or buds, so that healthy explants can be obtained for subsequent propagation.

A breakthrough in heat therapy of grapevine was achieved at the University of California, Davis, in the early 1960s (GOHEEN *et al.* 1965). Vegetating plants were exposed to 37—38 °C, shoot tips 1—5 cm long were removed after 4 weeks or more and rooted on a heated (25 °C) sand bed under mist or, as recently proposed, on agarized nutrient medium under sterile conditions (OTTENWAELTER *et al.* 1973).

This procedure is now widely utilized throughout the world, with local adaptations. In our laboratory, heat treatment is performed using 2-year-old potted plants or unrooted cuttings buried in sand and placed directly in the hot chamber (SISTO and MARTELLI 1965). Shoot tips as short as 0.5 cm are excised after 30, 60, 90, 120 and 150 days, to be rooted under mist. Rooting percentages range from about 30 % to over 70 % according to the cultivar and to the *Vitis* species (V. SAVINO, unpublished information).

In a recent improvement of this technique reported from California, individual buds from candidate vines are grafted into healthy LN-33 rooted cuttings and are heat-treated for 60 days (GOHEEN and LUHN 1973). The shoots grown from such buds were found to be virus-free in 77 % of cases, a figure considerably higher than that (28 % of virus-free shoot tips) obtained with standard procedures (GOHEEN and LUHN 1973).

Another method worth mentioning has been developed in France by GALZY (1964). Pieces of green shoots or mature canes, 2—3 cm long with a terminal bud, are surface sterilized and placed in glass tubes containing agarized nutrient medium. The explants are grown first at 20 °C for 2 months under artificial illumination, then for 3 months at 35 °C. After heat treatment, survivors are again multiplied aseptically on nutrient medium *in vitro* prior to transplanting in pots in a glasshouse.

An interesting combination of *in vivo* and *in vitro* treatments is being investigated in France (A. VUITTENEZ, personal communication) and in Spain (AYUSO and PENA-IGLESIAS 1976). With this procedure, potted vines are subjected to ordinary thermotherapy (37 °C) for 1—3 months. Apical meristems (0.2—0.3 mm) are then excised from these plants and are "grafted" into green cuttings of healthy rootstocks cultured *in vitro*. This technique is laborious and graft take is not always very high. Nevertheless, although definite results are not yet available, it may prove valuable for the elimination of those pathogens, like the agent of yellow speckle, that have resisted heat therapy so far.

In conclusion, heat therapy seems to be at present the only available tool for establishing virus disease-free material when no healthy stocks can be found in nature. Not only the technique is sufficiently reliable and simple to apply but it has proved beyond doubt not to cause irreversible disturbances to grapevines. Thus, it can be safely used for the improvement of current sanitary standards of modern viticulture.

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Eingegangen am 6. 2. 1979

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