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ACTION FA1003
East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding

ACTION FA0807
Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Phytoplasmas and viruses management in Grapevine Collections for Germplasm Conservation, Mobilization and Evaluation

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Grapevine germplasm collections should be considered with a particular respect by the phytosanitary services. They represent a precious source of genetic resources. Not rarely a cultivar is maintained just in one collection.

We have not data for what concerns the sanitary status of the grapevines germplasm collections, but following a general impression, viruses, phytoplasmas and some other bacteria represent a real risk for their exploitation and to a certain extent to their survival.

So a sustainable strategy for germplasm conservation and evaluation should include diagnostic protocols and disease control. Moreover the germplasm mobilization would represent a real opportunity to reduce the risk of loosing biodiversity. In this context specific protocols for germplasm circulation among collections, including specific quarantine procedure management, and more in general for the for phytosanitary management of the grapevine repositories should be developed to favor the healthy germplasm conservation.

Index

Status of *Vitis* germplasm conservation in Europe

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Seeing that grape cultivars and the wild grapevine were severely threatened, at the end of the 1970s, researchers initiated measures to safeguard the grapevine genetic resources. Now it is exactly 30 years ago, that in Thessaloniki (29 April - 1 May 1982) a Working Group on Vitis Genetic Resources reviewed the status of Vitis germplasm preservation in Europe, a meeting organized by the International Board for Plant Genetic Resources (IBPGR, today called Bioversity). In the same year the Organisation for Vine and Wine issued a resolution on the "Collection and conservation of the genetic resources of Vitis ssp." (OIV Resolution N° 2/82). Collecting missions, maintenance of cultivars, breeding lines and wild species in grapevine collections, cooperation and free exchange of genetic material between the collections and the implementation of an international database were recommended. Since then in all European wine growing countries numerous activities have been initiated to prospect, describe and preserve old autochthonous cultivars, their clones and the wild species Vitis vinifera L. subsp. sylvestris. In addition four projects on grape genetic resources (Genres081, Black Sea – Project, GrapeGen06, COST FA1003) and the ECPGR Vitis Working Group bundled the national activities and moreover pushed to common efforts like the use of the FAO/IPGRI Multi Crop Passport Descriptors (MCPD), standardized characterization according to agreed descriptors of mainly autochthonous cultivars, genotyping of accessions by 9 recommended SSR-markers, trueness to type assessment, on farm evaluation and maintenance, inventory of Vitis sylvestris populations and the documentation of data in a sort of virtual crop specific network, the European Vitis Database.

MCPD data from 31.856 accessions located in collections of 22 countries have been uploaded into the European Vitis Database until April 2012. The number of accessions maintained by each of the 34 collections concerned is given. In comparison to the number

of cultivars hold by same institutes 25 years ago, an enlargement in size can be stated and thus more genetic resources are preserved.

The present status report describes the progress achieved in the last 30 years.

With respect to the medium term objective of germplasm monitoring and safety duplication of Most Appropriate Accessions (MAAs), suggestions are made regarding detection of unique accessions and tagging of neglected and endangered cultivars. The usefulness of the European Vitis Database for that goal is outlined. In that context the work carried out by the world largest grapevine repository in Vassal is demonstrated. The curators integrated use of ampelography and genotyping to ascertain trueness to type and the assignment of the variety number of the Vitis International Variety Catalogue (VIVC) to almost all accessions is exemplary. Variety numbers assist to assemble identical accessions/cultivars independent from the cultivar name (synonymy and homonyms). This beneficial work should be indicative for all of us.

The European Vitis Database is reflecting Vitis germplasm conservation in grapevine collections. In order to draw a realistic and up to date picture about the status of Vitis germplasm conservation in Europe in addition a review from each country would be needed encompassing the following issues: (a) existence (%) of old vineyards (or mixed plantings) offering highly variable germplasm, (b) number of autochthonous cultivars (which originated in the country - estimation), (c) number of minor cultivars, (d) number of neglected and endangered cultivars existing in grapevine repositories only, (e) occurrence of Vitis sylvestris in wild habitats, (f) realization of prospections, (g) preservation of clones, (h) existence of national organisms linking collections and activities and (i) Vitis germplasm related projects or programs. For the 2nd meeting of the ECPGR Vitis Working Group in September 2012 at the Institut für Rebenzüchtung Geilweilerhof corresponding country reports will be prepared by the national representatives of the Vitis Working Group.

Grapevine virus diseases: impact and advanced diagnosis of associated agents

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Grapevines are infected by sixty-two viruses, among which some members of the families *Secoviridae, Closteroviridae* and *Flexiviridae* are the most important, as they can induce yield losses in excess of 60% and/or seriously affect the plant survival in the field. *Grapevine fanleaf* and *Arabis mosaic* viruses, *Grapevine leafroll-associated* viruses and *Grapevine virus A*, respectively associated to infectious degeneration, leafroll and rugose wood complexes, are considered a serious threat in all the major wine growing areas of the world and their control mainly consists in the adoption of virus-controlled plant propagation material (Martelli and Boudon-Padieu. Options Méditerr., Ser. B, Stud. Res. 55, CIHEAM, 279, 2006). The primary viral inoculum derives, by and large, by the uncertified planting material used for the establishment of new vineyards, within which the secondary spread is operated by ectoparasitic nematodes and mealy bug vectors. Thus, propagative materials, regardless of whether for commercial or scientific purposes, should strictly conform to the phytosanitary rules issued by the EU Directive no. 2005/43/CE, which defines what can be called a "minimal" sanitary status, and was amended in Italy by the stricter D.M. 24/6/2008.

Preventing virus spread requires effective and robust diagnostic methods for their detection, which relies on a well-established panel of biological, serological, and/or molecular techniques. Currently available laboratory protocols allow to obtain results from a relatively large number of samples in few days and some methodologies, i.e. ELISA, represent a simple diagnostic platform accessible to minimally equipped laboratories, without loosing in robustness and sensitivity. Either serological or the majority of molecular techniques reveal known and well characterized viruses, whereas approaching unknown diseases requires to implement "broad-spectrum" strategies of

detection. Available tools are represented by "generic PCR" (Saldarelli *et al.*, Eur. J. Plant Pathology 104: 945-950, 1999) which permits to identify viruses at the genus/family level with degenerate primers targeted to conserved viral sequences, and DOP-RT-PCR (Rott and Jelkmann, Eur. J. Plant Pathol. 107: 411– 420, 2011) mainly starting from replicative dsRNA form of unknown viral genomes. Recently, innovative approaches in metagenomics allowed the characterization of viral communities in a vineyard or in individual vines, and resulted in discovery of new viruses (Giampetruzzi *et al.*, Virus Research 163: 262–268, 2012) whose role in disease induction, either alone or in synergism with other viruses, is under study. These next generation technologies of sequencing, deeply exploring the genomic contents of cells/tissues and organism, open a new era in the assessment of the phytosanitary status of a plant as well as in analysing plant/virus interactions.

Phytoplasmas associated with grapevine yellows diseases: an overview

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Phytoplasmas are obligate intracellular bacterial parasites restricted to the phloem sieve elements of the infected plants and are transmitted by phloem-sucking insects belonging to the families *Cicadellidae*, *Cixidae*, *Psyllidae*, *Delphacidae* and *Derbidae*. They are associated with diseases of several hundred plant species, including some economically important crops. Grapevine yellows (GY) is a worldwide disease complex associated with genetically different phytoplasmas. GY-affected *Vitis vinifera* shows leaf enrollment accompanied by yellowing or reddening, rubbering of the canes and desiccated clusters. Epidemiology of the different GY diseases, undistinguishable based on symptoms observation, strictly depends on the involved phytoplasma because of the insect-vector specificity and their behavior. GY diseases are attributed to infections by at least nine distinct phytoplasmas. In Europe, "flavescence dorée" (FD) and Palatinate grapevine yellows (PGY, present only in Germany), are associated with phytoplasmas classified in the ribosomal group 16SrV, while "bois noir" (BN) is attributed to phytoplasmas classified in stolbur group (ribosomal subgroup 16SrXII-A). In Australia, Australian grapevine yellows is associated to '*Candidatus* Phytoplasma australiense' (ribosomal subgroup 16SrXII-B), and to '*Ca.* P. aurantifolia' (ribosomal group 16SrII). Grapevine yellows in Virginia is associated with a '*Ca.* P. asteris'-related strain (ribosomal group 16SrI-A) and X-disease group (ribosomal group 16SrIII) phytoplasmas. In Chile '*Ca.* P. fraxini' was also associated with GY together with stolbur and 16SrI-B and 16SrI-C phytoplasmas. In Italy and in South Africa '*Ca.* P. asteris' (16SrI-B) was associated with severe GY epidemics as well. In order to distinguish each GY from the others, an important research topic focuses on

developing molecular tools for specific phytoplasmas identification. In Europe, the employment of such methods for the certain exclusion of FD and BN phytoplasmas from grapevine certified propagating material is becoming urgent. PCR-based techniques allowed development of useful tools for the identification of phytoplasmas; standard protocols include nested PCR amplification of phytoplasma 16S rDNA using universal or group specific primers and RFLP analyses in order to determine the taxonomic (ribosomal group/subgroup) affiliation. Further molecular characterization, performed by sequence analyses on genes less conserved than 16S rDNA, found additional markers useful for developing suitable analytical tests for faster and specific detection of FD and BN phytoplasmas. Up to now, innovative molecular approaches developed to this aim are: (i) Real Time PCR and reverse transcription – Real Time PCR for the detection of phytoplasmas associated with FD and BN; (ii) nanobiotransducer for detecting FD phytoplasmas; (iii) multiplex nested PCR for simultaneous identification of FD and BN phytoplasmas; (iv) Ligase Detection Reaction (LDR) DNA microarray to detect and distinguish FD and BN phytoplasmas.

Furthermore, multiple gene sequence analyses (Multi Locus Sequence Typing, MLST) on ribosomal (*rplV-rpsC*) and non ribosomal (*secY*, *map*, *uvrB*, *degV*, *hlyC*, *vmp*, and *tuf*) genes highlighted an unexpected genetic heterogeneity among both FD and BN phytoplasma populations, identifying different FD and BN phytoplasma strains that can be associated with specific ecological niches (plant hosts, insect vectors, geographic origin). MLST analyses improved the chance to associate phytoplasma-specific molecular markers with biological features, opening new perspectives for the studies of FD and BN epidemiology.

Insect vectors of grapevine phytoplasmas in Europe

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Phytoplasmas are transmitted by phloem-feeding insects (order Hemiptera). In Europe, grapevine's phytoplasmas belong to the groups: 16SrV, agents of "flavescence dorée" (FD); and 16SrXII, agents of "bois noir" (BN). Mixed FD and BN infections within the same plant may also occur.

The vector of FD is *Scaphoideus titanus*, a nearctic species now widespread in Europe. It is monophagous on grapevine, and transmits FD directly from grape to grape. *S. titanus* is a quarantine pest, and is a target for mandatory sprays in many European countries; among the active ingredients used, neonicotinoids seem to be the most effective. However, other insects may be able to transmit FD to grapevine. *Dictyophara europaea*, often carrying 16SrV phytoplasmas, is able to transmit them from *Clematis vitalba* to grapevine under laboratory conditions; *D. europaea* lives on many weeds; however, adults are seldom found on the grapevine's canopy. Another species, *Orientus ishidae*, has been recently associated to FD in Europe, but its vector role has yet to be proven; *O. ishidae* is polyphagous on many trees and shrubs, and adults are frequently found on the grapevine's canopy.

The only known vector for BN is *Hyalesthes obsoletus*, that can occasionally feed on grapevine as adult, whereas nymphs live on the roots of weeds, where they can acquire phytoplasmas. In north-western Italy, it develops mainly on *U. dioica*, and only rarely on bindweed (*Convolvolus arvensis*); the latter, along with *Calystegia sepium* and *Ranunculus bulbosus*, is an important host plant in Germany; *Lamium orvala* is also a recognized host. BN is transmitted from weeds to grapevine, which may be considered a dead-end host. No insecticidal treatments are recommended against *H. obsoletus*, which can be controlled by agronomical practices and push-and-pull strategies. Other cixiids, frequently

found in the vineyards, resulted positive to BN: *H. scotti*, *H. luteipes*, and *Reptalus quinquecostatus*; the latter was also able to transmit BN to an artificial feeding system.

At the DIVAPRA laboratories different activities are carried out to uncover critical aspects in the study of insect vectors of phytoplasmas to grapevine. Molecular biology techniques are applied to the specific identification of some vectors, when it is extremely difficult, if not impossible, to discriminate different species only based on morphological features. In the field of grapevine yellows (GY) vectors, in the last years protocols based on PCR-RFLP analysis were set up, permitting to discern different cixiid species, four belonging to the genus *Reptalus*: *R. quinquecostatus*, *R. melanochaetus*, *R. cuspidatus*, and *R. panzeri*, and three to the genus *Hyalesthes*: *H. obsoletus*, *H. luteipes*, and *H. scotti*. Such protocols allow to extend the collecting period to the whole year by permitting to identify juveniles and females, and open to in-depth studies on the associations between single species and host plants.

Another promising topic covered by the research group is the study of the symbiotic *microbiota* of insect vectors, which has increasing relevance as it opens innovative perspectives for containing the vector populations by means of symbiotic control strategies. Recent investigations on the symbiotic bacteria of *S. titanus* and *H. obsoletus* underlined as bacteria of the genus *Asaia* are dominant within the microbial community of *S. titanus* and colonize different organs, including salivary glands and reproductive organs, where also the phytoplasma is located. Furthermore these symbionts are able to be vertically and horizontally transmitted, permitting a rapid spread among insect populations. Such a feature makes these cultivable bacteria good candidates for a paratransgenic approach for employing them in the control of phytoplasma transmission. Other interesting primary symbionts have been observed in different organs of *H. obsoletus*, including '*Candidatus* Sulcia muelleri', '*Ca.* Purcelliella pentastirinorum' and the newly discovered '*Ca.* Vidania fulgoroideae'.

Recently new achievements on vector sampling have been obtained. Marking and re-capture techniques were applied to study *S. titanus* flight activity and dispersal. Moreover geostatistics and artificial neural networks were used to study the influence of some environmental factors on *S. titanus* populations.

Grapevine crown gall: an old, emerging disease

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Crown gall is considered one of the most important and widespread bacterial diseases of grapevine (*Vitis vinifera* L.) throughout the world. It is known in Europe for more than 150 years and can be still of great phytopathologic significance in the vineyards and nurseries, especially in cold-climate regions. The disease is predominantly caused by tumorigenic strains of *Agrobacterium vitis*, more rarely by tumorigenic *A. tumefaciens* and *A. rhizogenes*. Unlike *A. tumefaciens* and *A. rhizogenes*, that are broad-host-range pathogens, *A. vitis* is specific to grapevine. Crown gall reduces vigor and yield of grapevines and severe disease may cause partial or complete death of infected plants. High losses occur in nurseries where different graft combinations with visible symptoms are unmarketable and must be discarded. Typical symptoms of crown gall are tissue proliferation (tumors) formed mostly on the lower areas of the trunk and on aerial canes. Tumorigenic and nontumorigenic strains of *A. vitis* are also able to cause specific root decay and it has been hypothesized that both types may be factors involved in the "replant disease" syndrome. Wounds mainly caused by freezing temperatures or grafting serve as a crucial entry points for the pathogen and its complex infection process. During the infection process DNA fragment from the bacterial tumor inducing (Ti) plasmid is transferred and integrated into the plant genome (interkingdom gene transfer). This leads to the overproduction of the phytohormones auxin and cytokinin, resulting in an uncontrolled proliferation of plant cells and tumor formation. *A. vitis* is unevenly distributed within systemically infected grapevines and able to survive in vineyard soil, particularly in the vicinity of infected plants and in their debris. Another important aspect

is the ability of the pathogen to be latently present within the grapevine, providing an important means of spread over short and long distances by asymptomatic propagation material. Management of grapevine crown gall is not easy considering that no effective chemical control measures are available. However, production of *A. vitis*-free grapevines is an essential prerequisite for an effective prevention of the disease, and great efforts should be done in this direction. For this reason, shoot tip propagation of grapevine and thermotherapy are available as control measures. Planting of crown gall and cold-resistant cultivars and rootstocks would be a good practice when establishing new vineyards. Biological control of crown gall is another promising approach in the control of the disease and several antagonistic bacterial strains have shown a certain level of efficiency in preventing tumor formation. Indexing of grapevines for the endophytic presence of *A. vitis* is a very important preventive measure.

Differentiation and identification of tumorigenic strains can be rapidly assessed by PCR using primer combinations specific for bacterial Ti plasmid and chromosomal genes. However, a high level of genetic diversity among *Agrobacterium* strains may limit the efficiency of PCR. In our studies *virC*, *virD*, *virF*, *pehA* and 23S rRNA gene-specific primers (Bini *et al.*, Vitis 47:181, 2008; Pulawska *et al.*, Syst. Appl. Microbiol. 29:470, 2006; *Suzaki et al.*, J. Gen. Plant Pathol. 70:342, 2004; Szegedi and Bottka, Vitis 41:37, 2002) were reliable in routine detection and identification of a broad range of *Agrobacterium* strains occurring in grapevine. However, there is necessity for development and standardization of indexing procedures including protocols of analysis and sampling methods. In the EU and many other European countries, *A. vitis* is not listed as a quarantine pathogen and is considered as a "quality organism" which significantly reduces the value of propagation material. Therefore, the importance of proper phytosanitary measures in grapevine nurseries and on commercial lots should be emphasized.

Validation of diagnostic protocols for the detection of grapevine viruses covered by phytosanitary rules

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The Italian Ministry of Agriculture has financed the Finalized Project "ARNADIA" with the purpose to produce validated reference diagnostic protocols for the control and monitoring of plant pathogens of phytosanitary interest. The grapevine viruses covered by phytosanitary rules were identified among them. To this end, it has been established the "Working group ARNADIA – grapevine viruses (WG)" which included 8 Research Institutions, 3 accredited Private Laboratories, one Plant Health Service and one Association of Grapevine Nurseries.

The aim of WG was to produce reference and validated serological and molecular protocols allowing for the harmonization of the diagnosis of 8 grapevine viruses (GLRaV 1, 2, 3, GVA, GVB, ArMV, GFLV and GFkV).

A protocol validation is the evaluation of a process to determine its fitness for a particular use. A validated assay yields test results that identify the presence of a specific target. Parameters that influence the capacity of the test result to predict accurately the infection status of the sample are diagnostic sensitivity (ability of the method used to detect the presence of the pathogen in the samples surely infected by the pathogen in

question - true positive) and diagnostic specificity (ability of the method used to not detect the presence of the pathogen in samples not infected by the pathogen in question - true negative). Other parameters that must be considered and which determine the efficiency of a protocol are: the analytical sensitivity (the smallest amount of infectious entities that can be identified by the diagnostic method), repeatability (degree of conformity of the results obtained in replications of the method, made at short intervals of time, using the same reference sample and in the same working conditions i.e. equipment, operator, laboratory) and reproducibility (degree of conformity of the results obtained using the same method with the same reference samples in different laboratories).

In this view, 122 grapevine samples (varieties, rootstocks and pool) have been analyzed by serological (using 24 antisera of three commercial companies) and molecular (multiplex RT-PCR) protocols. Moreover, different extraction methods, reagents and materials have been compared in 13 laboratories. Processing of the obtained results (about 24,000 data) has led to the definition of validation parameters according to UNI/ENI/ISO 16140 and 17025 and EPPO standards PM7/76 and PM7/98.

ELISA has proved to be a highly effective technique comparable with the molecular method, although the latter, as expected, it turned out to be more efficient for some viruses and on specific samples (rootstocks and pool). On these bases, serological and molecular protocols could be considered as alternative methods and their use has been suggested for different specific applications.

All results and parameters obtained will be the subject of detailed discussion.

Management of grapevine gene-banks and prevention from virus infections

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In experimental vineyards, the spread of virus diseases mediated by vectors or by human activities causes relevant economic damages, waste of time and can adversely affect research work. The knowledge of grapevine virus epidemiology and a preliminary assessment of the risk of infection or reinfection taking into account the local pedoclimatic conditions, constitute the basic information for working out a suitable prevention system. Even if grapevine is the crop with the highest number of viruses species reported, only some of them, responsible of the three most dangerous virus diseases (grapevine leafroll, infectious degeneration and rugose wood complex), are widespread and efficiently transmitted by not-flying vectors such as nematodes, mealy bugs, soft scale insects and of course the man.

Fortunately the transmission through seed and pollen don't seem to create any particular problems to the activity of breeders and furthermore the sexual reproduction could be considered as an alternative procedure of sanitation. The control of nematode populations, despite the limited number of species vectoring the European and Mediterranean *Nepoviruses* (mainly *Xiphynema index* for GFLV and *X. diversicaudatum* for ArMV) and the persistent highly specific transmission, is difficult; the direct chemical control through nematocide or fumigation is proved to be ineffective in open field and therefore only preventive measures can be adopted, aimed to avoid the introduction of nematodes from infested surrounding vineyards or, in case of replanting, to check the presence of the vector and eventually to eradicate the nematode population and the alternative plant host species. Among the insect vectors the mealy bugs, being mobile

and able to transmit with a semi-persistent and low-specific mechanism in all the development stages and furthermore having more generations, are certainly more dangerous than soft scales; even is some insecticides are effective in chemical control, due to the high efficiency in transmission reported with not evident and low infestation levels, the populations must be continuously and carefully monitored especially when favorable climatic conditions occur. However very often the reinfection or over-infection of the plants in germplasm collections is directly caused by misguided human activities in particular through the illegal hidden exchange among Countries of unchecked, often infected, propagation material, the over-grafting frequently used in the evaluation plots to test the new crosses or the unconscious dangerous introduction of new pathogens and vectors. Proper facilities and plans for implementing preventative measures, should be at hand before the establishment of a new planting (e.g. choosing suitable locations, using particular agronomic solutions for vineyard's establishment) or during its management, e.g. for controlling vector populations. A rational strategy that comprises various types of intervention for keeping unmodified the original sanitary status of the vines must be economically sustainable, thus related to the value of the material and to the purposes of conservation and research. Although different solutions can be proposed for varietal gene-banks, parental or collection vineyards, primary sources of certified clones, evaluation fields of new crosses, in no case the risks of virus infection should be overlooked and an effective integration of viticultural and phytopathological competences should always be sought for optimizing the work of researchers, germplasm collection managers, breeders and selectors.

Elimination of viruses, viroids and phytoplasma from grapevine germplasm

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According to recent reports, 60 viruses have been documented as infecting grapevine: this is far greater than the number of viruses known in any other single perennial crop. Some of these grapevine-infecting viruses are highly damaging and widespread. Grapevine is also one of the most permissive, natural viroid hosts, and five species, belonging to different genera, have been isolated from grapevine plants up to now. In addition, other severe grapevine diseases are associated with phytoplasmas: "flavescence dorée" (FD) and "bois noir" (BN). Phytoplasmas have spread in many viticultural areas of North Italy causing severe damages and strong concerns among grape growers, phytopathologic services and nurseries. These phytopathologic problems not only can affect the yield (as for quality and quantity) of commercial vineyards and the production of propagation material, but also can represent a risk for the sanitary status of grapevine germplasm collection. The percentages of virus-infected vines are often very high for minor cultivars, due to centuries of grape culture on the same plots and the presence of the natural vectors. For all those intracellular pathogens, once a plant is infected there is no cure available for field-grown plants. This makes essential the propagation of virus-free sources only and, in case, the application of sanitation techniques to obtain healthy mother plants in the frame of germplasm management as well as selection programs.

Thermotherapy (*in vitro* or *in vivo*) and meristem tip culture are by now reliable and efficient techniques for virus eradication. While meristem culture is particularly effective in eliminating phloem-limited viruses, thermotherapy is normally required for the elimination of other viruses such as *Nepoviruses* that readily invade plant meristems. Although results can be quite variable depending on the number and type of viruses, starting material, skill of personnel and other factors, these techniques are regarded as routine procedures for virus eradication. Somatic embryogenesis, which is usually

adopted to regenerate plantlets in biotechnological breeding programmes, has also been employed to successfully eradicate viruses from grapevine, despite the specific limits that still hamper its use.

At the Grugliasco Unit of the Plant Virology Institute (CNR, Italy) 59 clones of 35 grapevine cultivars (for a total of over 300 individual lines) have been sanitized from viruses in the last years through *in vitro* thermotherapy, meristem culture and, recently, somatic embryogenesis. Nearly all these cultivars are minor varieties that belong to the rich biodiversity of several Italian regions: Piemonte, Valle d'Aosta, Liguria, Emilia-Romagna, Campania, Calabria. Indirect somatic embryogenesis has shown excellent potentiality in virus eradication particularly when traditional approaches did not provide satisfactory results. The grapevine rupestris stem pitting-associated virus (GRSPaV) resulted very difficult to be eradicated through meristem tip culture, *in vivo* and *in vitro* thermotherapy, since more than two-thirds of derived lines were still GRSPaV-infected after treatment; regeneration through somatic embryogenesis always gave rise to plants free from GRSPaV. Eradication of grapevine leafroll-associated virus-1 (GLRaV-1), grapevine leafroll-associated virus-3 (GLRaV-3), *Grapevine virus A* (GVA) and *Grapevine fleck virus* (GFkV) was obtained with a 100% success percentage, while this percentage was 94% for the *Grapevine fanleaf virus* (GFLV). Somatic embryogenesis also showed high efficiency in elimination of viroids: two widespread viroids, grapevine yellow speckle viroid 1 (GYSVd-1) and hop stunt viroid (HSVd), were never detected in embryo-derived plantlets of four grapevine cultivars originally infected, even three years after their transfer to greenhouse conditions.

With regard to phytoplasmas, our experience supports the hypothesis that FD phytoplasma does not infect efficiently the *in vitro* cultured grapevine plants, and that micropropagation itself can be considered for eradication attempts from infected materials. Additional strategies for phytoplasma elimination, such as addition of antibiotics (50 mg l-1 oxytetracycline) to the culture media, are advisable particularly if the BN phytoplasma is present.

Grapevine propagation material movement and related phytosanitary rules in the EU

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The phytosanitary regulation in force in the European Union is very cautious as far as the genus *Vitis L.* is concerned, due to the important role of grapevine culture in both the economy and the European agricultural tradition and its phytopathological vulnerability, as testified by the dramatic events recorded in the past. Phytosanitary issues within the European Union are regulated by Directive 2000/29/EC. In the Directive, the list of harmful organisms related to viticulture includes the bacterium *Xylella fastidiosa*, agent of Pierce's disease, leafhoppers vectors of the disease and the generic definition of "non-European grapevine viruses" (list "A1"). The "A2" list includes the grape phylloxera (*Daktulosphaira vitifoliae*), which is not present in limited areas of the Mediterranean basin, and the bacterium *Xylophilus ampelinus*. Moreover, *Margarodes* and the phytoplasma of the grapevine "flavescence dorée" must not be present in the grapevine plants.

An updated list of quarantine organisms for Europe and the Mediterranean basin is included in the PQR (Plant Quarantine data Retrieval system), a practical database managed by the EPPO.

Facing the aforementioned threats, the EU banned the import of grapevine plants and parts of grapevine plants from all non-EU countries. The import of grapevine plants in the EU, which is not allowed for commercial purposes, is however allowed for the purposes of research and genetic improvement, though it must comply with the procedures included in Directive 2008/61/EC. The Directive establishes the conditions under which quarantine, essential for post-entry testing, is managed. The Directive also regulates the conditions for the release and use of imported material in the EU. Licensing procedures and structural and organizational provisions for quarantine stations are included.

Further regulation is now provided by the International Standard for Phytosanitary Measures (ISPM-FAO) No. 34 "Design and operation of post-entry quarantine stations for

plants", issued in 2010. Directive 2008/61/EC constantly refers to the FAO/IBPGR Technical Guidelines for the Safe Movement of Grapevine Germplasm, issued in 1991.

The Guidelines, as far as grapevine is concerned, include:

- safe movement of hardwood cuttings or green cuttings only, or rather *in vitro* cultures;
- material origin controls, collection and dispatch procedures;
- dormant cutting treatment with hot water thermotherapy;
- further disinfection, insecticide and fungicide treatments;
- indexing.

The first "short term" indexing step, as described by the Guidelines, was based on the knowledge available in 1991 and includes visual tests, indexing with herbaceous plants, ELISA tests, classic bacteriology methods and genetic tests which are now rather obsolete (sPAGE, dsRNA analysis, nucleid acid hybridization).

The second "long term" indexing step is based on wood grafting with indicator grapevine plants (*Vitis riparia* GdM, *V. rupestris* SG, K5BB, Cabernet franc, Pinot noir and others).

Both indexing steps are considered crucial, even though the material has already been treated with thermotherapy, a method with acknowledged but partial efficiency. In the event of positivity for one or more viruses, healing through meristematic shoot tip culture is suggested. The whole aforementioned procedure takes a very long time to be performed (from three to seven years).

The procedure is in force today, and must therefore be followed.

Still, a review of the procedure could well be advisable, given the availability of new detection techniques. PCR and total RNA sequencing in plants (BLAST etc.), which are relatively cost-limited, could well be an excellent alternative to the use of indicator plants, since they are equally reliable but timesaving and cost-cutting. The information acquired through these diagnosis tools, broadened to a large platform of harmful organisms, might well prevent an indiscriminate use of thermotherapy.

The latter requires careful examination: its impact on the community of grapevine-related organisms cannot be assessed a priori as it unpredictably affects the reaction capacity of grapevine plants, the inner ecology of which is too little known today.

Grapevine collections free from pathogens: tools and their application

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The grapevine collections are very important tools to maintain grapevine biodiversity and historical germoplasm as well however in several cases especially grapevine from poor cultivated or non commercial varieties could be infected by several graft transmissible pathogens such as viruses, phytoplasmas and other systemic bacteria. In the majority of the cases these pathogens are not inducing evident symptomatology in short time after grafting therefore the possibly infected material of collection could represent a dangerous pathogen reservoir.

In order to control pathogen presence in already made collections and to prevent the spreading of the above pathogens together with the grapevine germplasm to other collections. Then, it is mandatory to exclude presence of quarantine pathogens such as "flavescece dorée" (FD) phytoplasmas and advisable to exclude relevant pathogens for quality such as viruses and phytoplasmas agent of "bois noir", by using the most sensitive detection techniques available. It is advisable however to acquire any possible information concerning the phytosanitary status of the circulating grapevine material in order to prevent possible unforeseen outbreak of disease such as those occurred for FD disease when a grapevine insect such as *Scaphoideus titanus* (previously named *Scaphoideus littoralis*) was introduced in Europe. It is known in fact that a high number of different phytoplasmas are able to infect grapevine worldwide in the presence of appropriate insect vector or by grafting or micropropagation techniques application and crown gall is an old severely remerging disease at least in the major viticultural areas of EU and US.

First step before transferring germplasm among collection must be the verification of their sanitary status taking into account that tests to verify virus and bacteria presence

should be carried out preferably during winter/spring time while those to detect phytoplasmas are more sensitive in Summer and Fall periods and the most sensitive techniques such as ELISA and PCR must be employed.

In the case of germplasm having no clean plants available after the survey it is necessary to clean the material using thermotherapy and or shoot tip culture in order to eliminate the pathogens. These techniques are not eliminating the pathogens from all the produced material therefore molecular tests are again necessary to assess the grapevine health status before the material can be employed for collection and/or field dissemination. In case of virus or phytoplasma infected grapevine germplasm of unique genetic value it must be maintained under insect proof condition while it is infected in order to avoid contamination of other germplasm in the same collection. In the same way the clean germplams should also be protected in insect proof environment in order to avoid its recontamination. It is also very important to keep the collection clean from insect that are virus (mealy bugs and scale insects) or phytoplasma vectors (leafhopper and cixiids) and also the soil must be clean from *Agrobacterium tumefaciens* and collection should be protected from frost or mechanical damages increasing crown gall dissemination.