TRANSGENIC RESISTANCE: STATE OF THE ART AND PERSPECTIVES

M. Fuchs

Institut National de la Recherche Agronomique, Unité Mixte de Recherche Vigne et Vins d'Alsace, INRA/Université Louis Pasteur, Laboratoire de Virologie, 28 rue de Herrlisheim, 68021 Colmar, France.

Introduction

Grapevine viruses can cause severe losses by substantially reducing yield, affecting fruit quality, and shortening the lifespan of infected plants in the vineyard. Strategies to control viruses are essentially preventive with sanitary selection programs or directed to eradicate virus vectors with agrochemicals. These strategies are effective at limiting the presence of viruses in propagation material and reducing the infectious potential of vectors. However, their practical impact is somewhat limited if new plantings are established in areas with a long history of monoculture of grapes. Moreover, concerns have been expressed on the use of agrochemicals used to control virus vectors, and even restrictions applied, because of their acute toxicity and adverse effects on the environment.

Due to the severity of viral epidemics, the difficulties of implementing efficient control strategies, and the increasing demand for sustainable and environmentally-safe viticulture practices, there is a great need to develop varieties that are resistant to viruses. Virus resistance has not been achieved in elite varieties yet because genes for resistance have not been found or the few sources of resistance that have been identified are difficult to transfer by conventional breeding. Genetic transformation is an attractive alternative for genetic improvement of grapes because it potentially elevates the shortcomings of conventional approaches by allowing the insertion of specific virus resistance traits directly into desirable elite varieties.

Development of transgenic grapevines for virus resistance

The advent of biotechnologies, and the concept of pathogen-derived resistance in particular (18), opened new avenues in the mid 80s for the development of virus-resistant grapevine varieties. Since the pioneering work of M.G. Mullins and colleagues who were the first to stably transform grape plants (16), several reports described the development of transgenic grapevine and *Nicotiana* plants with virus-derived gene constructs. P. Gugerli and M. Laimer da Camara Machado and colleagues were the first to report on the development of transgenic *Nicotiana* expressing various gene fragments from *Grapevine fanleaf virus* (GFLV) or *Arabis mosaic virus* (ArMV) at the 11th ICVG Meeting in Montreux, Switzerland (19, 21). Thereafter, numerous studies described the development of transgenic grapevines or herbaceous hosts of grapevine viruses, expressing gene constructs from GFLV, ArMV, *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 3* (GLRaV-3), among other viruses of interest. Since the 13th ICVG Meeting in Adelaide, Australia, the development of transgenic grapevines (6, 8, 9, 10, 14, 22, 23, 26), including some engineered for resistance to viruses (1, 5, 7, 11, 12, 13, 17, 20, 24) or fungi (3, 27), and transgenic *Nicotiana* engineered for resistance to grapevine viruses (13, 15, 17, 20, 28) has been described in the literature.

Integration of virus-derived genes in transgenic grapevines and resistance evaluation

Virus-derived genes, mainly the coat protein (CP), movement protein or RNA dependent RNA polymerase genes, have been engineered and transferred into the genome of rootstocks, *V. vinifera*, or interspecific hybrids as complete, truncated, sense, anti-sense, translatable, or untranslatable versions (1, 5, 7, 11, 12, 13, 17, 20, 24). Also, genes with potential to protect against fungi have been integrated into grapes (3, 27). Research is on going to engineer resistance to crown gall and phytoplasms.

Reaction of transgenic material to virus infection was essentially evaluated by mechanical and graft-inoculation with herbaceous or woody cuttings in the case of *Nicotiana* and grapevines, respectively. Noteworthy, high levels of resistance were reported against GFLV (15), ArMV (20), GVA (13, 17), GVB (13), and *Grapevine berry inner necrosis virus* (28) in *Nicotiana*. Also, encouraging resistance data from the first field trial ever with transgenic rootstocks expressing the GFLV CP gene were presented by Fuchs and associates at the 13th ICVG Meeting in Adelaide, Australia (4).

Environmental safety issues

Environmental safety issues have been expressed on the large-scale release of transgenic plants, including grapevines. Such issues are particularly relevant in the case of a perennial crop like grape because it is grown for many years in the field, thus increasing the probability of occurrence of unintentional phenomena such as recombination, heteroencapsidation, complementation, transgene dissemination through pollen flow, etc. Identifying risks and assessing their impact on the environmental is a necessary prerequisite for the safe deployment of virus-resistant transgenic grapevines. It is also a wise approach to develop sustainable and environmentally-friendly viticulture practices.

The occurrence of heterologous encapsidation was demonstrated in transgenic *Nicotiana benthamiana* and *N. occidentalis* expressing the CP gene of GVA and GVB, respectively, which were challenged with the heterologous virus, as well as in co-infected nontransgenic *Nicotiana* (2). Will such observations hold true under conditions of natural virus infection in the vineyard? Further research is needed to answer this question. Interestingly, however, the only risk assessment study performed so far in the field with transgenic grapevines suggests no detectable environmental impact beyond natural background events regarding the emergence of recombinant GFLV species (24).

Perception and acceptance

Social and ethical concerns have been expressed on the use of transgenic grapevines, sometimes creating a strong climate of opposition. In France, the controversial acceptance and general confusion on the usefulness of GFLV-resistant transgenic grapevines prompted the Director of INRA to take a novel and unique initiative in 2001 (http:www.inra.fr/Internet/directions/SED/science-gouvernance/ITA-Vignes/index.html). This initiative was based on a wide consultation and the promotion of pro-active and transparent dialogues with stakeholders. Thus, representatives of the scientific community, grape growers, nurseries, environmental protection agencies, and the public at large were invited to debate on the legitimacy and relevance of research activities on transgenic grapevines engineered for resistance to GFLV. Another topic of discussion was the legitimacy and necessity of testing transgenic grapevines in the field. This unique experience lasted almost for two years and called for a strong support of research in this controversial field. However, it did not consider favorably any commercial release of transgenic grapevines in the near future.

Future directions

Encouraging levels of resistance have been reported in transgenic grapevines, mainly against GFLV (24). More research is needed now to assess the sustainability and stability of the engineered protection. However, based on the successful protection of herbaceous hosts with the same transgenes as those expressed by transgenic grapevines (13, 15, 17, 20), there is little doubt that transgenic grapevines exhibiting high levels of resistance to viruses will be obtained. In addition, the recent advances in unraveling gene silencing and the synthesis of siRNA (small interfering RNA) should provide new tools for engineering stable and durable protection against viruses.

Risk assessment studies are needed with transgenic grapevines. Preliminary information on the occurrence of heterologous encapsidation in *Nicotiana* plants (2) highlights the need for more research in this field. Will the data obtained with transgenic herbaceous plants hold true with transgenic grapevines? Will data from laboratory experiments translate into similar findings in the field? Along the same line, it will be interesting to see whether future experiments will confirm that virus-resistant transgenic grapevines are likely to have limited or no detectable environmental impact (24, 25).

Will the trueness-to-type and clonal fidelity need to be verified in transgenic material? Such verification may be asked for in the future in transgenic wine grape cultivars.

Will virus-resistant transgenic grapevines be made available to growers? It is difficult to realistically envision any commercial release of virus-resistant transgenic grapevines in the near future. However, the severe detrimental impact of viruses, the strong demand for a reduction in the reliance on toxic agrochemicals for virus vector control, the pledge for a safe and sustainable viticulture, and the success of biotechnologies at offering alternatives to current control strategies, there is a wide open window of opportunity for practical use of virus-resistant transgenic grapevines within a reasonable period of time, given education, dialogue, and promotion of informed choices.

Concluding remarks

In the early 90s, grapevine was considered more or less recalcitrant to transformation and regeneration. Thus, limited studies reported on the development of transgenic grapevines. However, once the development of embryogenic cell cultures was optimized and transformation protocols well established, publications on the development of transgenic grapevines became more numerous.

Significant progress has been achieved over the past three years on the development and evaluation of transgenic grapevines. This is evidenced by the numerous publications and reports in this field (more than 25 from 2000 to 2003). During this period, major breakthroughs have been reported on the techniques to transform, select, and regenerate transgenic grapevines, including some developed for virus resistance. There is no doubt that the next three years will be as exciting, if not more, as we can anticipate valuable information on the persistence of engineered traits and the level of resistance. It is also reasonable to anticipate important information on the usefulness of the virus-resistant transgenic material and its compatibility with environmentally-friendly and sustainable production methods. Significant progress is also likely to be achieved on the acceptance of virus-resistant transgenic grapevines through scientifically-based constructive initiatives for dialogue.

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ENGINEERING DURABLE RESISTANCE IN GRAPEVINES: A NOVEL STRATEGY FOR INTEGRATED DISEASE MANAGEMENT TO OVERCOME ENVIRONMENTAL IMPACT OF PESTICIDES

R. Fischer and S. Schillberg

RWTH Aachen, Institute for Biology VII, Worringerweg 1, 52074 Aachen, Germany
email: schillberg@molbiotech@rwth-aachen.de

Despite current efforts, the maintenance of pathogen-free grapevine stocks is challenging. Even if pathogen-free planting material is available, these plants can be re-infected in the field. Control of the virus itself by chemical protection measures is difficult, environmentally unfriendly and expensive.

Transgenic plant technology offers the possibility to make grapevine inherently resistant to pathogen infection. Our rationale is to generate anti-pathogen antibodies that inactivate major grapevine pathogens. In a 4-year project funded by the EU (5th framework), we will generate biologically safe, virus-resistant plants through expression of such recombinant antibodies. Because of their economic importance for viticulture, we focus on the nepoviruses *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV), on the closterovirus *Grapevine leafroll-associated virus 2* (GLRaV-2) and the ampelovirus *Grapevine leafroll-associated virus 3* (GLRaV-3) as the target pathogens.

Isolated viruses or bacterial produced viral proteins have been used to immunize animals and to isolate specific antibodies or antibody fragments by hybridoma or phage display technology. After 18 months project time monoclonal antibodies and scFv antibody fragments binding to ArMV or GFLV virus particles and GLRaV-3 coat protein have been identified. Selection of additional antibodies binding to viral replicases or movement proteins is in progress. Identified antibody fragments will be cloned into plant expression vectors and used for transformation of somatic embryonic anther material to generate virus resistant grapevine.

EU project partners:

Prof. Rainer Fischer, RWTH Aachen, Institut fuer Molekulare Biotechnologie, Worringerweg 1, 52074 Aachen, Germany

Dr. Gabi Krczal, Staatliche Lehr- & Forschungsanstalt fuer Landwirtschaft, Weinbau & Gartenbau, Centrum Grüne Gentechnik, Breitenweg 71, 67435 Neustadt, Germany

Prof. Giovanni Martelli, University of Bari, Department of Plant Protection and Applied Microbiology, Via G. Amendola n. 165/A, 70126 Bari, Italy

Hans-Joerg Boehm, Viveiro Jorge Boehm Lda. (Plansel), Quinta de S. Jorge, 7050 Montemor-o-Novo, Portugal

Project homepage:
www.molbiotech.rwth-aachen.de/grapevine.htm

VIRUS RESISTANCE BREEDING IN GRAPEVINE

M. Laimer

Plant Biotechnology Unit, Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Nussdorfer Lände 11, 1190 Vienna, Austria

The Plant-Biotechnology Unit at the IAM is involved since many years in resistance breeding via the pathogen mediated approach against viruses (1). The soil-borne nepovirus *Grapevine fanleaf virus* (GFLV) is the main causal agent of the grapevine fanleaf disease, one of the most damaging and widespread viral diseases affecting grapevine. This nepovirus, together with other nepovisurses, can cause rapid death of young plants or a gradual decline over several years (infectious degeneration). The virus is spread via both propagating material and the nematode vectors *Xiphinema index* and *X. italiae*. Some Middle-Eastern *V. vinifera* and *Vitis* species belonging to the *sub-genera Muscadinia* showed a good level of resistance to *X. index* and/or to GFLV, but this does not completely exclude viral infection (2, 3). New truly resistant grapes has not been developed yet with traditional breeding methods (4).

Virus resistance breeding in grapevine is limited since resistance genes either do not exist or are not yet known. In an attempt to induce virus resistance (5) we introduced nine different constructs containing chimeric coat protein (CP) genes of grapevine fanleaf virus (GFLV), either the full-length CP, 3 truncated forms of the CP and 2 untranslatable forms of the CP gene either in antisense (AS) or sense (S) orientation (6), the ArMV CP gene (7), grapevine virus A (GVA), and grapevine virus B (GVB) (8), first into the herbaceous host *Nicotiana benthamiana* and *N. occidentalis*, respectively, and subsequently into embryogenic cultures of *Vitis vinifera* (Russalka cv. - selfpollinated) (9), 110 Richter (*Vitis rupestris x Vitis Berlandieri*) (10), the cultivars Nebbiolo CVT230, Lumassina and Blaufränkisch (11) by *Agrobacterium*-mediated transformation. Putative transformed tissues were selected by continued proliferation on kanamycin containing medium. Selected embryos were germinated and entire plants regenerated and acclimatized to greenhouse conditions. Integration of the different CP sequences was confirmed by PCR and Southern blot analyses. Data obtained in herbaceous model plants indicate that all constructs are able to confer resistance to a different degree. Confirmation in woody plants is currently underway, but can only be assessed in final field trials.

However, several problems have to be solved before transgenic grapevines can be utilized as actual solution to the virus problems, including technical (low rates of embryogenesis and embryo germination in many cultivars, low transformation efficiency, need of selectable markers alternative to antibiotic resistance, use of site-specific or inducible promoters) and social aspects (biosafety and public perception of genetically modified organisms).

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THE CONSTRUCTION OF GENE SILENCING VECTORS FOR THE INTRODUCTION OF MULTIPLE VIRUS RESISTANCE IN GRAPEVINES

J.T. Burger, M. Engelbrecht and C.Van Eeden

Department of Genetics, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa.

Introduction

Grapevine viruses constitute some of the most serious threats to the South African wine industry. Leafroll disease is widely acknowledged as the most important virus disease of grapevines, while the rugose wood complex is seen as an emerging virus disease.

Posttranscriptional gene silencing is a natural process during which steady state levels of mRNA are diminished by targeted degradation. It holds great promise as a technology to introduce virus resistance in plants. Gene silencing has been studied extensively under different descriptive names in a number of organisms: co-suppression or posttranscriptional gene silencing in plants (1, 2), quelling in fungi (3) and RNA interference (RNAi) in nematodes (4), insects (5), protozoans (6), and mice (7). The mechanism for gene silencing is not fully understood, but current hypotheses hold that the cascade of events is triggered by the presence of dsRNA, complementary to an endogenous gene or to exogenous RNA like a virus or an intron. An RNase III (Dicer) cleaves the dsRNA into 21-23 bp small interfering RNAs (siRNAs), which in turn are bound within an RNA-induced silencing complex (RISC). After an ATP-dependent conversion to a single strand conformation, siRNAs recognise and bind to homologous RNA after which an endonuclease cleavage of the target RNA occurs (8, 9).

By expressing dsRNA complementary to gene fragments of grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine rupestris stem pitting-associated virus (GRSPaV) in grapevine, we aim to introduce resistance to these two viruses. Towards this goal, we constructed a number of gene silencing vectors that were based on the generic plant gene silencing vector pHannibal (10). Expression of the gene silencing cassette of this vector yields an mRNA containing sense and antisense copies of the gene to be silenced, separated by a plant intron. Nucleotide homology will favour the formation of a stemloop structure, while the loop will be removed during post-transcriptional processing of the RNA, yielding dsRNA to trigger gene silencing.

Materials and Methods

Gene fragments from South African isolates of GLRaV-2 and -3, and GRSPaV were amplified by RT-PCR using primers with 5'-extensions containing restriction sites to facilitate subsequent cloning. A portion of the green fluorescent protein (GFP) was isolated by PCR from the plasmid vector pBIN mGFP5-ER (11), also using primers containing restriction sites. Individual PCR products were ligated into the intermediate vector pGEM-T-Easy, and sequenced. Gene fragments of GLRaV-2 (490 bp), GLRaV-3 (481 bp) and GRSPaV (610 bp) were liberated from their respective pGEM-T-Easy vectors by restriction digestion, gel-purified, and cloned into corresponding sites in the 5'- and 3'-multiple cloning sites (MCS) of the pHannibal vector, in sense and antisense orientations. Tandem vectors were constructed by ligating gene fragments (GLRaV-3/GRSPaV and GLRaV-2/GFP) using shared restriction sites on the ends of these fragments. Tandem constructs were ligated in sense and antisense orientations into the 5'- and 3'-MCSs of pHannibal. All silencing cassettes were removed as a *Not* I fragment from pHannibal and ligated into a *Not* I site of the plant expression vector, pHellsgate (10). *Nicotiana benthamiana* plantlets were regenerated after agrobacterium-mediated transformation with the different constructs. Agro-infiltration experiments were done as described (12).

Results and Discussion

A number of silencing vectors were designed and constructed. Since resistance to GLRaV-3 and GRSPaV is our ultimate aim, "single" vectors, i.e. containing individual gene fragments to these viruses were constructed. To be able to test our hypothesis in tobacco (neither GLRaV-3 nor GRSPaV infect *N. benthamiana*), a "single" vector for GLRaV-2 (which does infect *N. benthamiana*) was also made. The 5'-portions of the coat protein (CP) genes of these viruses, which generally show a higher level of nucleotide homology among different isolates, were chosen as target sequences for these vectors. A "tandem" vector, containing fused CP gene fragments of the two viruses, was constructed by cloning sense and antisense fused fragments into pHannibal. This "tandem" cassette was subsequently cloned into the plant expression vector pHellsgate. Once again, for testing in tobacco, a "tandem" construct of GLRaV-2 and GFP was made. This construct was agro-infiltrated into *N. benthamiana* plants that constitutively expressed GFP, to test the ability of this vector to transiently silence GFP expression in these transgenic plants.

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INDUCTION OF SILENCING IN TRANSGENIC TOBACCO (*N. BENTHAMIANA*) AND GRAPEVINE (*VITIS* SPP) PLANTS

G.M. Reustle[1], R. Jardak-Jamoussi[2], R. Ebel[1], C. Burkhart[1], M. Becker[1], R. Wolf[1], T. Manthey[1], A. Bassler[1], T. Wetzel[1], A. Ghorbel[2] and G. Krczal[1],

[1]Centrum Gruene Gentechnik, SLFA Neustadt, Breitenweg 71, D-67435 Neustadt / W, Germany
[2]Institut National de Recherche Scientifique et Technique, Laboratoire d'Adaptation et d'Amélioration des Plantes, BP 95, Hammam-Lif. 2050, Tunisie.

The major virus disease in German viticulture is the Fanleaf disease, caused by a group of nepoviruses, the *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV) and *Raspberry ringspot virus* (RRV). The viral infections dramatically decrease the value of an infested vineyard because of the decrease in both the yield and quality of the grapes. Due to a lack of natural genetic resources for virus resistance, suitable for cross breeding programs, a transgenic approach was chosen to develop rootstocks resistant against these nepoviruses. As the viruses are transmitted by nematodes only, resistance of rootstocks against these viruses would be sufficient to prevent infection of the grapevines. To establish constructs possibly inducing gene silencing, highly conserved sequences (movement protein of GFLV and ArMV, 3` non coding region of RNA-2 of RRV) from the viruses (1) were combined with defective interfering (DI)-sequences from Potyvirus and / or used to clone inverted-repeat constructs. The constructs were genetically transferred in tobacco (*N. benthamiana*) and grapevine rootstocks (*Vitis* sp.) by *Agrobacterium* mediated transformation. Selection of transgenic tobacco plants were carried out by using *nptII* (kanamycin resistance) or PAT (Phosphinotricin resistance) as selectable marker genes. For grapevine transformation, the PAT selectable marker gene was use. Evaluation of the constructs in tobacco (*N. benthamiana*) by challenge inoculation with the relevant viruses yielded transgenic lines showing immunity, recovery, retarded infection and susceptibility. Preliminary analysis for siRNA using the virus derived sequence as a probe, could detect siRNA in the resistant lines. Further analysis are in progress.

For genetic engineering of grapevine, embryogenic tissue of rootstocks (SO4, 125-AA, 5C, Binova) was induced from anther cultures and used for *Agrobacterium* (LBA 4404) mediated transformation. After 16 to 20 weeks of selection on phosphinothricin (PPT) containing media, newly generated somatic embryos were harvested form the original explants and cultivated on PPT free media for regeneration. Transgenic grapevines could be regenerated from these embryos and were propagated and maintained *in vitro.* Analysis of the regenerated lines by PCR showed high number of escapes and chimeric character of the plants. Repeated propagation of putative transgenic lines by one node cuttings and PCR analysis of the therefrom obtained plants yielded non-chimeric transgenic grapevines. Molecular analysis showed low copy number in the transgenic lines (1 to 3 copies), siRNA analysis are in progress.

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RNA INTERFERENCE AS A NOVEL STRATEGY FOR RESISTANCE AGAINST *GRAPEVINE FANLEAF VIRUS*

C. Bruno[1], P. Arce[2] and P.D. Valenzuela[1]

[1]Fundación Ciencia para la Vida e Instituto Milenio de Biología Fundamental y Aplicada, Av. Marathon 1943, Santiago, Chile.
[2]Depto. Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile.

Grapevine fanleaf virus is one of the most important and widespread virus that affect *Vitis* genera, causing important economic losses due to the reduced quality of grape and productivity of vineyards.

The absence of natural resistance to this virus has carried to the development of pathogen-derived resistance strategies. Generally the strategies applied to induce pathogen-resistance can be divided in two groups: one that induce resistance by the expression of viral genes in plants (1, 2), and the second, at RNA level by directly interfering with viral replication (3). In the second group RNAi appear as a novel mechanism of defence against pathogens in many organism, from the worm *C. elegans* to plants, as *A. thaliana*, and human cells. In latest model RNAi has been efficiently used to induce resistance to many viruses (4), in plants RNAi has been mainly used in functional genomics, nevertheless has been recently applied to produce plants resistant to crown gall tumorigenesis (5) and to *Cucumber mosaic virus* (6).

Grapevine fanleaf virus is a (+) polarity virus, of the nepovirus groups. Its genome is encoded in two single stranded RNA and, in some isolates, an additional satellite RNA. We chose three regions in the RNA2, varying in length from 300 to 500pb, corresponding to a gene responsible for its replication, the movement protein and the coat protein, respectively. These regions were amplified by PCR from a cDNA library of a Chilean isolate of GFLV (GFLV-Ch80) that has been completely sequenced, using primers designed in conserved domains between the Chilean isolate and the isolates F13 and NW. The amplified products were cloned in the binary vector pHellsgate2 (7), which has been specifically designed for RNAi. This vector contains the sequence for homologous recombination allowing directional cloning using the Gateway system (Invitrogen), to produce the cloning as inverted repeat separated by an intron sequence of 1600pb. The inserts were sequenced and *Agrobacterium tumefaciens* was transformed with these vectors for the transformation of *Nicotiana benthamiana* plants, which will be challenged with the virus to study the efficiency of this strategy to induce virus-resistance. Analysis of the potential of this approach is in progress.

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REGENERATION OF TUNISIAN TRANSGENIC GRAPEVINE PLANTS AND EVALUATION OF THE GENE CONSTRUCT FOR *GRAPEVINE FANLEAF VIRUS* RESISTANCE

R Jardak-Jamoussi[1]*, B. Bouamama[1], G. Krczal[2], A. Mliki[1], T. Wetzel[2], G.M. Reustle[2] and A. Ghorbel[1].

[1] Laboratoire d'Adaptation et d'Amélioration des Plantes, INRST, BP: 95, Hammam-Lif 2050, Tunisie.
[2] Centrum Grüne Gentechnik (CGG), SLFA Neustadt, D- 67 435 Neustadt/W, Germany
*Tel : +216 71 430 855, fax : +216 71 430 934, e-mail: rjardak@yahoo.fr

Grapevine fanleaf virus (GFLV) is a nepovirus causing important and severe damages in Tunisian vineyards. Genetic engineering of grapevines offers the opportunity to induce resistance against this virus. In a first step, a transformation system using PAT-GUS-Intron construct, the *gus* (ß-glucuronidase) as a reporter gene and the *bar* gene for Basta herbicide resistance (PAT: Phosphinotricine acetyltransferase), was used to transform anther derived embryogenic calluses of a Tunisian variety (Arich dressé). The effect of this marker at different concentration (1; 2.5; 5 and 10mg.l-1PPT: phosphinotricine) was studied on the grapevine embryogenic callus development.

In a second step, a construct including a conserved sequence of the movement protein of a Tunisian GFLV isolate cloned in inverted repeat orientation was elaborated for virus resistance. Its evaluation on the tobacco model system (*Nicotiana benthamiana*) was carried out by challenge inoculation with GFLV. Transgenic lines showed immunity, recovery, delayed infection and susceptibility. At present, molecular analysis are in process in order to look for small RNAs as markers for sequence specific gene silencing.

For grapevine transformation, embryogenic callus were inoculated by *Agrbacterium tumefaciens* (LBA4404) and selected for 24 weeks on medium containing 2,5mg.l-1 PPT. This concentration of PPT was lowered to 1 mg.l- in the 8 following weeks. Newly arising embryos were transferred to PPT free medium and induced for conversion and plant regeneration. Using PCR analysis, 15% of the regenerated grapevines were found to be transgenic. Nowadays, our efforts are focused on evaluating resistance of this transgenic grapevines ex vitro against GFLV.

EVALUATION OF TRANSGENIC GRAPES TOLERANCE TOWARD *GRAPEVINE FANLEAF VIRUS*

I.Tsvetkov[1], B.Choleva [2], M.Yankulova[1], N.Minchev[2], V. Colova[3] and A.Atanassov[1]

[1]AgroBioInstitute, 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria, e-mail: ivantsvetkov@hotmail.com
[2]Sofia University, Faculty of Biology, 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria
[3]Florida A&M University, Tallahassee, Florida 32307, USA

Introduction

Grapevine fanleaf virus (GFLV), transmitted by soil ectoparasitic nematodes *Xiphinema index* and *X. italiae* (5) is one of the most widespread and economically important diseases of the grapevines. A common way for field control of GFLV is based on the use of agrochemicals against above-mentioned vectors. Because of its environmental toxicity and reduced effectiveness, the use of nematocides is restricted in many countries (1,2). Genetic engineering offers an alternative way for development and evaluation of virus-resistant grapevines. The aim of this study was to evaluate the tolerance of transgenic grapevines carrying the GFLV coat protein gene to transmission of the virus by *X. index* vector, under pot-plant greenhouse conditions.

Materials and Methods

Transgenic lines of seedless genotype 7-3/2 E1 (self- pollinated Rousalka 3) and rootstock *Vitis rupestris* cv. Rupestris du Lot carrying the gene of coat protein of GFLV were developed as previously described (7). After nematode multiplication in potted fig trees (supposed to be free of GFLV), about 2000 adult nematodes *X. index* were isolated. Such nematodes were transferred to pot-rooted cuttings of GFLV diseased *V.vinifera* cv. Pamid, used as virus donor. Taylor *et al.* (3, 4) described a suitable period between 120 and 180 days for the *Longidoridae* nematodes to be virus infected from donor plants. Twenty nematodes were isolated, after 180 days development. and used for electron microscopy analyses for GFLV detection inside the nematodes, as described by Trugill et al. (6). The virus transmission experiment was carried out with 1-year-old pot-rooted transgenic and control plants of both, the rootstock *V. rupestris* cv. Rupestris du Lot and the seedless genotype 7-3/2 E1. Each variant included 4 transgenic and 4 control potted plants. Eighty infected adults of *X. index* were added in each pot. Results were observed after 120 days by serological test on experimental plants for infection with GFLV, using DAS- ELISA kits obtained from BIOREBA®.

Results and Disscussion

Experiments with *X. index* nematodes, carrying GFLV showed, that transgenic plants of the seedless genotype 7-3/2 E1 did not become virus infected where as all control plants became infected (Table 1). All tested *V. rupestris* cv. Rupestris du Lot plants, both transgenic or controls were negative. This could be due to a particular nematode resistance of the rootstock or short invasion time. The greenhouse and field transgenic plants exhibited no visible morphologic differences compared with the controls. Results from this greenhouse test indicate that transgenic grapevines carrying the GFLV coat protein gene possess a promising level of tolerance to GFLV. Additional field evaluation of the transgenic lines in naturally infected vineyards are in progress.

Table 1. Results from DAS- ELISA test for detection of Grapevine Fanleaf Virus in experimental plants

Samples		ELISA (405 nm)
Vitis rupestris cv. Rupestris du Lot	Control plants	0.060 (0.037-0.081)*
	Transgenic plants	0.039 (0.029-0.051)*
7-3/2 E1 (selfpollinated Russalka 3)	Control plants	2.141 (1.857-2.203)*
	Transgenic plants	0.060 (0.036-0.082)*
Virus donor plant		1.988
Positive control		1.557
Negative control		0.085

* range of photometric value

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ANTIBODY-BASED RESISTANCE IN GRAPEVINE: GENERATION, CHARACTERISATION AND EXPRESSION OF SINGLE CHAIN ANTIBODY FRAGMENTS SPECIFIC TO GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3

G. Nölke[1], M. Orecchia[1], P. Saldarelli[2], M. Dell'Orco[2], A. Minafra[2], G.P. Martelli[2], R. Fischer[1,3] and S. Schillberg[3]

[1]RWTH Aachen, Institute for Biology VII, Worringerweg 1, 52074 Aachen, Germany
email: noelke@molbiotech@rwth-aachen.de
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale CNR, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy
[3]Fraunhofer Institute for Molecular Biology and Applied Ecology, c/o RWTH Aachen, Institute for Biology VII, Worringerweg 1, 52074 Aachen, Germany

Recombinant antibodies (rAbs) have been produced in plants, retaining their biological characteristics to recognise the corresponding antigen. Pathogen-specific rAbs expressed in plant cells are an alternative approach to impair pathogen infectivity and to engineer resistance in the crops. However, successful use of antibodies to generate plant pathogen resistance relies on appropriate target selection, careful antibody design, efficient antibody expression, stability and targeting to appropriate cellular compartments.

We have chosen the coat protein of *Grapevine leafroll-associated virus 3* (GLRaV-3) as a target for generating specific scFv antibody fragments by phage display technology. In addition, we have cloned a scFv fragment from an existing hybridoma line producing a GLRaV-3 specific monoclonal antibody. The specific reactivity of the selected scFvs to GLRaV-3 particles and coat protein was demonstrated by ELISA. Since this virus is most vulnerable in the plant cell cytosol, the scFv cDNAs have been integrated into a plant expression vector enabling the accumulation of the antibody fragment in this compartment. Transient transformation of tobacco leaves by vacuum infiltration of recombinant agrobacteria has proven that scFv fragments are stable in the cytosol and retain their specificity to GLRaV-3. Transformation of grapevine is in progress and transgenic plants will be analysed for recombinant protein accumulation as well as improved resistance to GLRaV-3.

Generation of scFvs specific to viral proteins and their expression in grapevine could be a valuable molecular tool for integrated disease management.

INDUCTION OF GENE SILENCING TO *GRAPEVINE VIRUS A* AND *GRAPEVINE VIRUS B* THROUGH MARKER FREE TRANSFORMATION OF *NICOTIANA* AND *VITIS* SPP

C. Turturo[1], P. Saldarelli[1], G. Bottalico[1], M. Dell'Orco[1], A. Minafra[1], I. Gribaudo[2], V. Savino[1] and G.P. Martelli[1]

[1]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy.
[2]Istituto Virologia Vegetale del CNR, Unità Staccata Viticoltura, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy

Rugose wood is a complex disease of grapevine to which five different viruses are associated (1). In particular, the vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B (*GVB*)* are thought to be involved in the aetiology of Kober stem grooving and Corky bark, respectively, two of the syndromes of the complex. These viruses have a detrimental effect on the yield and survival of infected plants, and lower the graft take. Both viruses are spread in nature by mealybugs, which makes their containment difficult. Resistance to virus infections would therefore be desirable but, since natural sources of resistance to GVA and GVB do not seem to be available, the development of transgenic resistance could represent a possible strategy to pursue. Post transcriptional gene silencing (PTGS) has been recently identified as the mechanism that plants use against molecular parasites (2) and that underlies pathogen-derived transgenic resistance. A study was therefore initiated to evaluate the induction of PTGS to GVA and GVB using a transformation system that allows the generation of marker free plants.

Conserved coat protein (CP) sequences of GVA (200 nts) and GVB (184 nts) were amplified by RT-PCR from total nucleic acid (TNA) preparations of infected *Nicotiana* plants, and assembled in sense and antisense orientation in the vector pKannibal (3), to obtain the plasmids pKA and pKB. This cassette, driven by a Ca35S promoter, codes for a hairpin RNA (hpRNA) with a dsRNA in the stem and a plant intron in the loop regions; after self splicing of the intron a GVA or GVB dsRNA are expressed in plant cells. Potential protection from viral infection through PTGS induction, was evaluated in assays by biolistic inoculation of pKA and pKB DNAs to GVA-infected *Nicotiana benthamiana* and GVB-infected *N. occidentalis*.

The GFP gene in the binary vector pX6 (4) was substituted for pKA or pKB contructs to obtain plasmids pCreKA and pCreKB, respectively. Plasmid pX6 was shown to direct a site-specific DNA excision in transgenic *Arabidospis* plants through *Cre/loxP* mediated recombination. Expression of *Cre* recombinase is activated by an estrogen-based transactivator (XVE) and leads to the excision of a DNA fragment, containing the neomycin transferase II (NPTII) gene, between two *loxP* sites. Plasmids pCreKA and pCreKB and pX6 were used to transform *N. benthamiana* and *N. occidentalis* by *Agrobacterium*-mediated transformation. Grapevine transformation, using the same plasmids, was carried out on secondary embryos of cvs. Grignolino, Chardonnay, Nebbiolo and the ro ootstock 110R.

Co-inoculation of *Nicotiana* plants with pKA and pKB plasmids with GVA or GVB, showed a partial protection from viral infection ranging from 23% to 50% in the pKA/GVA and pKB/GVB combinations, respectively. These co-inoculated plants showed a delay in symptoms appearance or escape from infection, and preliminary experiments revealed the absence of viral RNAs in the upper non inoculated leaves, with respects to controls inoculated with vector pKannibal only.

Transgenic *N. benthamiana* and *N. occidentalis* plantlets were regenerated on a kanamicin-containing medium after *Agrobacterium* (LBA 4404) transformation. PCR analysis on genomic DNAs showed integration of the T-DNA of pCreKA, pCreKB and pX6. Molecular characterization of transgenic plants and evaluation of the DNA excision ability on estrogen (β-estradiol)-containing medium is under way. Grapevine secondary embryos and embryogenic callus were also co-cultivated with *Agrobacterium* and kanamicin selection was initiated.

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VIRUS ELIMINATION IN GRAPEVINE AND CROP PERFORMANCE

F. Mannini

Istituto Virologia Vegetale del CNR, Unità Staccata Viticoltura,Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy
E-mail f.mannini@ivv.cnr.it

Viral diseases are widely spread in grapevine and, although only some out of the 53 viruses found in *Vitis* (44, 45) have practical importance, nevertheless their presence in vine is generally considered detrimental. Thus severe and costly sanitary protocols are set worldwide in order to select and propagate 'virus-free' clones. The evaluation of the effects of viruses and related diseases however is not so easy as it could be thought (57). In the past, for example, the effects on yield were estimated by comparing the performance of symptomatic vs symptomless vines or, at the best, the virus diseases were tested by woody indicators. This approach is very much superficial because symptoms may be due to (or enhanced by) environmental conditions or the association of different viral agents. In addition investigations were seldom carried out on plants genetically uniform (i.e. the same grapevine clone with and without specific infection) adding a certain degree of genetic variability. In recent times, experiments are more correctly done comparing a given clone before and after heat-treatment or meristem culture and specific viral agents have been taken in account. Also in this case however it is difficult to discriminate the possible effect of the treatment itself or of the simultaneous elimination of unknown viruses. The comparison of vines before and after inoculation with a given virus, does not either guarantee from additional undesired viruses infecting the vines on trial. Finally, additional difficulties depend on the fact that evaluations carried out on a perennial crop like grapevine call for long term assessments and need a fairly high investments for setting experimental vineyards.

What said before makes quite clear why the virus impact on vine performance has not been investigated as much as necessary considering the importance of the matter and the fact that breeders and virologists after decades of clonal selection are still discussing if the genetic approach should prevail on sanitary aspects or vice versa (5, 31, 46, 56).

Although the knowledge of etiologic agents of these diseases has advanced, information on their effects on agronomic and enological performances of vines is still little and sometimes contradictory. Aware of the difficulty to generalise the findings of every single experiment, this report try to review the progress made in the field of the crop improvement by virus elimination focusing on the results obtained in the last decade. The aim is to give useful information, although not exhaustive, to breeders, nurserymen and growers.

Grapevine fanleaf (GFL), grapevine leafroll (LR) and rugose wood complex (RW), comprehensive of four distinct syndromes (RSP, rupestris stem pitting; KSG, Kober stem grooving; LNSG, LN33 stem grooving; CB, corky bark), are regarded up to now as the most harmful and widespread grapevine viral diseases. GFL is mainly induced by the infection of a Nepovirus, the *Grapevine fanleaf virus* (GFLV). LR is associated with different *Closteroviruses* (most of them have just moved to the new genus *Ampelovirus*) and, at present, two of them are considered the most significant in grapevine: *Grapevine leafroll-associated virus 1* and *3* (GLRaV-1 and GLRaV-3). In recent time however also GLRaV-2 is drawing attention of growers. The *Grapevine virus A* (GVA), often found in LR and RW affected vines, is a *Vitivirus* involved with the etiology of Kober stem grooving, as well as *Grapevine virus B* (GVB) which occurs in corky bark diseased vines. Among RW syndromes, rupestris stem pitting expression is correlated with the presence of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) of the *Foveavirus* genus. Finally, among potentially dangerous viruses it has to be mentioned the *Grapevine fleck virus* (GFkV), recently classified in the new genus *Maculavirus*. In grapevine are also present virus-like infections whose causal agents are still unknown as well as the practical damages they may cause to plants: vein mosaic (VM), vein necrosis (VN) and enations (EN).

Grapevine fanleaf

The disease and its aetiology are long time known. It is probably one of the most damaging in European vineyards, with symptoms ranging from malformations of canes to chrome-yellow discoloration, very often associated with vegetative vigour and crop reduction.

A vast literature (55) confirms the negative effects of GFL on yield, ranging from 20 to 90 % of crop reduction depending on the cultivar and the environment of culture. Sugars accumulation is often reported penalized too, although the strong reduction of yield may sometimes turn into some beneficial effects in wine quality (33).

A dramatic yield reduction (60 %) was related to deep alterations of photosynthetic apparatus in Chardonnay vines, experimentally infected by GFLV, in a field experiment carried out in Hungary (2). In Geneva NY, significant reduction in pruning weight, yield and bunch size was recorded in clonal plants of three *V. vinifera* cvs. graft-inoculated with different strains of GFLV and ArMV (*Arabis mosaic virus*) (29). ArMV, however, had milder impact on vine performance.

A long term field comparison between the same clones of the Italian wine grape cv. Nebbiolo, GFLV+GFkV-infected or GFLV+GFkV-free, showed dramatic differences in vegetative vigour (leaf area, shoot length, pruning weight, etc.) and yield (20-50%) in favour of virus-free plants (31, 32). The soluble solids accumulation in the berries increased until 20% whereas the anthocyanin content remained unaffected (43). Leaf morphology is also deeply modified by GFLV presence so different clones were better discriminated by leaf features when originally infected than after heat–treatment (37). This may have great practical implications on clonal identification, focusing the fact a clone may be correctly identified by its leaf morphology only when free from major viruses.

The GFLV exerts its detrimental effect especially on growth parameters then GFLV elimination is essential for rootstocks and the use of heat-treatment highly recommended in their selection. The cutting production from rootstock

mother-vines was reported highly reduced by GFLV (13) as well as the growth and the rooting of vines propagated by *in vitro* culture (1, 4, 20).

Two *Vitis vinifera x Muscadinia rotundifolia* hybrids (039-16 and 043-43) are recommended rootstocks in Californian sites infected with fanleaf degeneration: despite the fact of becoming infected by GFLV, the Cabernet sauvignon grafted on them did not show the reduction of crop registered on other rootstocks (54).

Fortunately nurseries are nowadays well conscious of GFLV dangers, so we may say the GLFV spread in the new plantings is getting more and more reduced over the years. Growers however must be aware of some possible changes in the aptitudes of healthy vines compared to the ones obtained from infected population. The GFLV-free vines, for example, have always higher vigour so cultural practices should be updated to face the growth increase.

In the cool climate of North-western Italy, the effect of different vine spacing (i.e. vine density/hectare) was studied with a heat-treated virus-free clone of Nebbiolo in order to optimize canopy management when vegetative vigour increases as a consequence of GFLV eradication. In this case a lower density (2900 vine/hectare) proved to be more promising than the traditional higher density (4300 vine/hectare) in terms of grape quality (soluble solids and berry colour were improved) thanks to the better canopy light interception (41).

Grapevine leafroll

Leafroll is probably the infectious disease most spread over the world and, although the complex of phloem-limited viruses associated to the symptoms were identified only in the eighties (23), the detrimental effects of LR was clear well before (19). Generally the damages caused by LR are less evident than the ones due to GFLV. The vines, although infected, usually have 'normal' vegetative growth and manage to bear a reasonable crop. The typical late-summer leaf reddening/yellowing and rolling symptoms are so widespread in aged commercial vineyards that they have became part of the landscape in many European areas and are accepted by growers as a normal expression of the plant morphology. This kind of general acceptance of LR presence and the consequent underestimation of the related damages make harder the control of this disease.

In the past, several field experiments comparing vines with or without LR symptoms, carried out in Europe as well as in California and Australia, indicated that symptomatic vines showed reduction in vigour, yield, bunch size, soluble solids and berry colour (55). The penalization of the vine performance was more or less important depending on the severity of symptoms, vintages, cultivars, etc.

In recent times similar trials were carried out more often on the same grape clonal material, LR-diseased by mean of graft-inoculation or LR-sanitised by heat-treatment. Mild LR had no effect on vine growth and yield but in some years reduced sugar accumulation (from 1 to 1.7 Brix°) when inoculated in Riesling and Zinfandel cvs. in California (58). The elimination by heat-treatment of a similar mild isolate of LR induced a 22 % increase of yield in the Australian Muscadelle variety without any modification in berry juice quality (47). Also in the cooler climate of France the artificial LR eradication induced 27 % higher crop in a Gewurztraminer clone but in this case soluble solids were slightly reduce compared to the control (3). In Australia, LR was reported to reduce skin colour in the fruit of Emperor table grape variety as well as sugar content (27).

Since the knowledge about the complex of viruses associated to LR improved, several trials were set checking not only the effect of the disease but also of the specific pathogenic agent with particular attention to the most spread GLRaV-1 and GLRaV-3.

In North-eastern Italy, GLRaV-3 infected clonal vines of Cabernet franc, Cabernet sauvignon and Merlot were compared to healthy ones showing a considerable loss in production (mainly in Merlot), due to lower number of bunch/vine and lower bunch weight. Grape qualitative parameters, controlled by berry sampling over the ripening period, showed that soluble solids and anthocyanin content were always lower in infected vines whereas titratable acidity was higher, with particular regards to malic acid (6,7). In Central Italy, healthy vines of Albana and Trebbiano romagnolo cvs. were inoculated with single and multiple infections: GFLV+GLRaV-3, RW+GLRaV-3+VM and RW+GLRaV-1+VM were the most detrimental on growth and yield (- 20 %) but had no effect on fruit maturity (14).

Lower sugar content and higher titratable acidity in the must resulted associated to a lower leaf net photosynthesis in GLRaV-3 infected vines of Spanish cv. Albariño (8). The elimination of mixed infections of GLRV-1+GVA and GLRaV-3+GVA from clones belonging to the cvs. Dolcetto, Grignolino and Nebbiolo in the cool climate of North-west Italy induced a slight increase of vegetative vigour and of leaf chlorophyll content in parallel with a great improvement of leaf photosynthetic efficiency, detectable since the time of fruit set (June) and well before the appearance of leaf symptoms (34, 40). The better physiological behaviour of healthy vines led to an increase of crop (around 30 %) without affecting sugar content, or to an increase of sugars (around 0.5-1 Brix°) with a similar amount of production (35, 42). It has to be focused that in the first case the virus involved was GLRaV-1 (+GVA) whereas in the second case was GLRaV-3 (+GVA). Accumulation of berry skin total anthocyanins was much faster and higher in plants set free from phloematic viruses. In conclusion the berry skin of healthy vines was more intensely coloured compared to infected plants confirming the deep interference of LR viral agents in the metabolism of phenols, and particularly of anthocyanins (24, 25, 36). The anthocyanin profile of berry skin remained unchanged between heat-treated or infected vines confirming to be a genotypic factor, but in healthy vines the content of all the different anthocyanins was much higher.

The improvement in grape soluble solids and phenol content resulted in beneficial effects on the quality of red wines. The results of the sensory evaluations elaborated by statistical ranking test (28) were more rewarding in the case of GLRaV-3 (+GVA) elimination: the wine obtained from healthy vines compared to the one from diseased vines was preferred in two out of the three vintages tasted (35). When GLRaV-1 (+GVA) was eliminated, on the contrary, the ranking test did not indicate any significant difference in taster preferences. However the preferences were given to the wine from healthy

plants in the two vintages when both virus-infected and virus-free vines were 30% bunch thinned (41). Higher yield at the same level of wine quality may be already a good result, however some cautions should be taken in cool climate environments not to exceed the threshold compatible with a good ripening of grapes. In this case bunch thinning should be wisely adopted to improve quality and the mentioned results indicate that with healthy plants the effect of cultural practices applied to improve grape quality is enhanced.

The findings obtained in Portugal with several local cultivars would suggest the negative effect of GLRaV-3 on grape yield may be quite moderate in warmer climate (30, 46). Mean crop differences between healthy and infected clones were far lower than the ones detectable between the genetically high-yielding or low yielding groups of tested clones. Also in the hot climate of Sardinia Island no significant penalization of crop and juice quality parameters was registered in own-rooted clonal selections, belonging to 5 local varieties, when healthy or affected by different mixed infections (among viruses the most present were GLRaV-3 and GFkV) (18). Only the average crop of the clones of the cv. Cannonau was 25 % reduced in presence of a virus 'cocktail' (GVA+GLRaV-1+GLRaV-3+GFkV).

It is known virus diseases contribute to increase the phenotypical variability within a vine population of a cultivar, however the results obtained with the cvs. Dolcetto and Nebbiolo confirmed the importance of genotype in determining clonal variability: comparative differences between pairs of virus-infected clones increased after virus eradication by thermotherapy regardless of the viruses involved (GLRaV-1, GLRaV-3, GVA) (38).

Another rewarding effect of GLRaV-3 elimination was registered with a clone of white Muscat. The heat-treated GLRaV-3-free clonal vines of this aromatic cultivar were much richer in soluble solids and in berry skin terpenes, despite the parallel yield increase, compared to GLRaV-3 infected controls (39).

Concerning GLRaV-2, it seems likely that a severe graft incompatibility between several *V. vinifera* cvs. and Kober 5 BB rootstock might be related to the presence of this *Closterovirus* (21). GLRaV-2 has been recently found in some popular certified French *V. vinifera* clones. According to the claimed milder symptomatology of this virus (at least in cool climate) and the good enological performance of these GLRaV-2-infected clones, their propagation and commercialization will continue in France (Boursiquot, p.c.).

In French–American hybrids GLRaV-3 is latent, however the absence of leafroll symptoms does not necessarily indicate their agronomic performance is unaffected by the virus. A research on Vidal blanc and St. Vincent hybrids grown in Canada indicates a 5 % reduction in berry weight and 5-9 % increase in juice titratable acidity. However the absence of differences in other yield components and in vegetative vigour suggests some tolerance of the two cultivars to the virus (26).

There is evidence of the negative implication of phloem-limited viruses in grape propagation. Scions collected from GLRaV-3+GVA infected or heat-treated mother-plants of a Nebbiolo clone gave 30 % more 'first class' grafted vines compared to control when bench-grafted onto healthy cuttings of Kober 5BB rootstock (35). The presence of GLRaV-1 or GLRaV-3 in mixed infections with KSG, RSP and VM in different rootstocks (420 A, Kober 5BB, Teleki 5A) caused over a 8 year period significant decrease (50 % on average) in the pruning weight of diseased vines (13).

The influence of GLRaV-1, GLRaV-3, GVA and RSP in single or mixed infections on micropropagation efficiency was investigated comparing infected and healthy meristem-derived plants of the same grape genotype. The *in vitro* plants obtained after sanitation produced more shoot/explant and longer shoots on proliferation medium (22) and longer roots on rooting medium (20).

The interaction between vine management (i.e. pruning, shoot thinning, etc.) and virus infection is far to be clearly investigated. LR effects (reduction of yield, vine growth and sugars) resulted amplified with minimal pruning of cordon trained vines of Cabernet franc in Australia (9), in the same environment light pruning enhanced expression of higher yield from clones of Sultana following thermotherapy for virus attenuation (10). Reducing or amplifying the detrimental effects of viral diseases by the severity of pruning has important consequences for viticultural practices.

Rugose wood complex

Rugose wood is a complex disease characterized by alteration of the woody cylinder affecting the movement of water and nutrients through the vascular systems of vines. The four different syndromes of rugose wood are very often associated with leafroll, as already mentioned in the previous paragraph, and it is not easy to elicit the specific effect of RW. Further investigations are then necessary to better understand the effect of RW alone, and particularly the vine performance modifications related to each single viral agent (GVA, GVB, etc.).

Significant reduction of vigour and yield were often reported associated to RW symptomatic plants (55) and there are indications that in hot climate the disease may lead to vine death (16, 49). The damages however are not always so evident and in cool climate often almost negligible so that, for instance, the certification protocol in Germany does not take in account the RW with practical implication on UE propagation material exchange.

A 8 year trial stated the highly detrimental effect of mixed infection KSG+RSP+GLRaV-3+VN on the growth of 420 A, Teleki 5A and Kober 5BB rootstocks (42-57 % reduction), whereas multiple infection KSG+RSP+GLRaV-1+VM caused similar effect on 420 A and Kober 5BB but did not induced any significant influence on Teleki 5A (13).

In Israel, a strong reduction of yield was reported in Thompson seedless when in presence of corky bark (50) whereas in Sardinia Island, although this RW syndrome is very much spread, the impact of the disease seems fairly light and it varies according to the different cultivars (17).

In Central Italy, Credi *et al*. (11) found a positive correlation between the intensity of RW symptoms and the reduction of yield, vigour and must soluble solids in a clone of the cv. Lambrusco grasparossa grafted on 13 clonal rootstocks. Among the tested rootstocks, Golia showed a good level of tolerance to the disease. These data confirm some kind of grape genotype tolerance to virus infection. Heat-treated healthy clones of Albana and Trebbiano romagnolo cvs. were infected using the same virus source vines of the previous experiment and their field performances controlled over 7

year period (14). The mixed infection with GLRaV-3 revealed a marked negative influence on yield (- 72.9 and - 46.6 % respectively) and growth (- 67.8 and - 39.1 %) on both the cultivars whereas the mixed infection with the GLRaV-1, inoculated only in Trebbiano, induced milder yield losses and growth reduction (- 21.2 and - 23.1 % respectively). In general no consistent differences in fruit maturity indices were observed between any of inoculated cultivars and healthy vines.

In the same experience the inoculum of a mixed infection of RSP+VN+VM had no significant effect on the field performance of the Albana clone. RSP and RSP+VM infections did not affect the field performance (pruning weight, yield, bunch weight, soluble solids, t. acidity, tartaric and malic acids) of two clones of Dolcetto grown in North-western Italy, as well as the wine quality assessed by sensory evaluation and ranking test (33). RSP infection both on *Vitis vinifera* and other *Vitis* species was deeply investigated in two cool environments of British Columbia (Canada). The results suggest that RSP had no major impact on grapevine growth and yield and it advanced fruit maturity in terms of lower titratable acidity and higher pH (48). RSP presence in vines of the Slovenian cv. Refozk, on the contrary, determined crop reduction and, quite surprisingly, a fairly high % of dead vines eight years after planting (52).

Minor infectious diseases (Fleck, Vein necrosis, Vein mosaic, Enations)

Some virus and virus-like agents cause latent or semilatent infections in *Vitis vinifera*. The most important are: fleck, vein necrosis, vein mosaic and enations. The practical implications of these diseases in commercial vineyards are still under discussion because experimental data on their effects on vines is really scarce.

Fleck (Fk), induced by GFkV, is symptomless in *V. vinifera* and in many American hybrid rootstocks, whereas typical symptoms are expressed in *Vitis rupestris*. *Vitis rupestris* S. George potted vines, when inoculated by one out of four Fk sources, gave woody material with reduced propagation aptitudes compared to control (i.e. number of rooted cuttings and number of Sangiovese cv. onto *rupestris* grafted vines) (53). Again a mixed infection of GFkV+VN+VM reduced the growth in rootstock mother vines of 420 A (51.9 %) and Kober 5 BB (37.1 %) although not of Teleki 5A (13, 15).

The negligible influence of Fk in *Vitis vinifera* seems to be confirmed by the results obtained on Albana and Trebbiano romagnolo cvs.: single infections of VM, VN and Fk, and a mixed infection of all of them did not affect significantly neither the yield nor the vigour (14). However a synergistic effect of a mixed infection of Fk and LR (named Ajinashika disease) was reported on Japanese wine grape cv. Koshu with a significant reduction of berry colour and soluble solids accumulation whereas the single Fk or LR infections were practically ineffective (51).

Field performance of healthy or VM-affected clonal plants of Chardonnay and Klevener cvs. were compared over 5 years without showing any differences in all horticultural parameters recorded (29). Furthermore no synergistic effect was noticed in VM+GFLV diseased vines.

Enation is a virus-like disease whose causal agent is still unknown. The typical symptomatology is not much frequent and fairly unstable but it has been observed in different viticultural areas all over the world. Trials carried out in the seventies reported severe reduction of yield in symptomatic vines in both cool or hot climates (55). The effect of enation was scientifically evaluated over a 4 year period by Credi (12) on Trebbiano romagnolo resulting in a crop reduction (13.2 to 23.0 %) according to the different expression of enation symptoms. Yield losses were higher in the symptomatic years and in the following one. However the growth rate, expressed as pruning weight, was not statistically significant.

Conclusions

Although not yet abundant, data on the improvement of grapevine crop performance due to virus elimination are getting consistent. Especially in the last decade, several trials were set in different environments and on different varieties collecting information on the matter. In addition the studies are taking more and more in account the specific viral agent and not only the symptoms, thus improving the comprehension of the complex virus-vine relationships. Up to now, field and cellar evaluations have confirmed the overall superiority of 'virus-free' plants compared to the virus-infected ones. The higher performance, due to the better efficiency of vine canopy, consists mainly in an increase of vegetative vigour and yield but, especially in cool climate environments, improvements of grape quality are also reported. A better grape ripening, i.e. higher content of sugars, anthocyanins (berry colour) and terpenes, was very often consequent to the elimination of phloem-limited viruses. There are evidences that such improvement of grape quality also turned into better wine characteristics.

Grapegrowers however must be aware of the modification in the behaviour of 'virus-free' vines (increase of vigour, yield, bunch size, etc.) and put into action a suitable vineyard management to optimize crop performance and grape quality (reduction of vine number/hectare, bunch thinning, severity of pruning, clone selection, etc.).

If the benefits of the elimination of the main harmful viruses (GFLV, GLRaVs, GVA, etc.) is out of discussion, the debate is still open on RSP and *V. vinifera* latent or semilatent infections (Fk, VM, VN) whose negative effects in terms of industrial viticulture are still not well demonstrated. The solution of these questions will have important implications on clone certification protocols in the UE, avoiding possible conflict between sanitary and genetic selection.

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ELIMINATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* FROM THE WINE GRAPEVINE MÜLLER-THURGAU (*VITIS VINIFERA* L.) THROUGH SOMATIC EMBRYOGENESIS

I. Gribaudo[1] , J. Bondaz[2], D. Cuozzo[1] and G. Gambino[2]

[1]Istituto Virologia Vegetale CNR, Unità Staccata Grugliasco-Viticoltura, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy
[2]Dipartimento di Colture Arboree, Università degli Studi di Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy

Grapevine leafroll disease is widespread in most viticultural areas, including Italy (1). The establishment of vineyards with vines free of grapevine leafroll associated viruses (GLRaVs) - as well as of the other most dangerous viruses of grapevine – is a control measure of paramount importance. Several methods have been applied to eradicate viruses from infected vines, including thermotherapy, meristem culture, fragmented shoot apex culture, chemiotherapy (2). Success varies with virus type and isolate, grape cultivar and specific approach. Somatic embryogenesis, a technique that is usually adopted to regenerate plantlets in biotechnological breeding programs (3), was shown to be highly effective in the elimination of GLRaVs (4, 5). Similar results were obtained in *Citrus* with the elimination of *Citrus* psorosis virus through somatic embryogenesis from stigma and style cultures (6). Grapevine fanleaf virus was eradicated when somatic embryogenesis was combined with thermotherapy (7). A reliable technique for the production of somatic embryos and regenerated plantlets from a large number of *Vitis vinifera* cultivars is the essential pre-requisite for the application of somatic embryogenesis as a sanitation method.

Anther and ovary cultures were initiated from a single plant of *Vitis vinifera* cv Müller-Thurgau infected by the *Grapevine leafroll-associated virus 3* (GLRaV-3). Inflorescences were collected from the vineyard when anthers became translucent yellow which corresponds to the microspore stage. The clusters were cold treated at 4 °C for 4 days, and sterilized by immersion for 10 minutes in a sodium hypochlorite solution (1.5 % active Cl) plus a few drops of surfactant. Somatic embryogenesis was obtained following the method of Franks *et al*. (8). All media contained Nitsch and Nitsch (9) mineral salts and Murashige and Skoog (10) vitamins. Explants were initially cultured on a callus induction medium with 6 % sucrose, 0.3 % gelrite, 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 8.9 µM 6-benzylaminopurine (BAP). The cultures (200 anthers and 40 ovaries) were maintained at 26°C in the dark.

Three months after the culture initiation, embryogenic callus that appeared was transferred to a different medium to favour the formation and proliferation of somatic embryos (8); this medium contained 6 % sucrose, 1 % agar, 0.25 % activated charcoal, 10 µM 2-napthoxyacetic acid (NOA), 1 µM BAP and 20 µM filter sterilized indole-3-acetic acid (IAA). Subsequently, single embryos were isolated and transferred on a medium with 3 % sucrose, 0.25 % activated charcoal, 1 % bactoagar and no growth regulator (embryo development medium). Germination was induced through two procedures: a) by prolonged culture with periodical transfers on fresh embryo development medium; b) by cutting the elongated embryos at the hypocotyl and culturing the shoot on a medium with 1.5 % sucrose, 0.9 % agar and 10 µM BAP. Ninety-nine embryos were subjected to germination procedure a) and 20 to procedure b). Subsequently, each plant derived from a somatic embryo was separately micropropagated on a growth regulator-free medium according to a previously described method (11), thus originating a single line.

The viral status of the mother plant and of regenerated plantlets was verify by subjecting samples to DAS-ELISA. Commercial ELISA kits (Agritest, Valenzano, I) were used for detection of GFLV, GLRaV-1, GLRaV-3, GVA and GFkV in the mother plant, and for GLRaV-3 only in regenerated plantlets. Antigen samples were extracted from wood material (scraped from pieces of dormant canes collected during winter pruning) for the mother plant, and from shoots (taken from *in vitro*-cultures) for regenerated plantlets. Tissues were macerated in extraction buffer (Tris-HCl, pH 8.2) at a dilution 1:10 (w/v). The reaction was assessed by measurement of absorbance at 405 and 490 nm. Thirty-eight embryo-deriving lines were tested (1 to 3 times each).

About 30 % of cultured anthers and 77 % ovaries produced callus. Within a four month-induction, somatic embryos developed asynchronously from 18.9 % of anther cultures and 28.6 % of ovary cultures. Germination of somatic embryos is often considered as the bottleneck of the entire procedure. Nevertheless, in the present experiment we obtained 59.6 % embryo germination with the procedure a) and 40 % with the procedure b).

ELISA tests confirmed the presence of GLRaV-3 only in the mother plant. All 38 embryo-deriving lines were ELISA-negative.

The regeneration protocol adopted in the present experiment previously proved to have good efficacy for several *V. vinifera* cultivars (12); the germination rate of Müller-Thurgau embryos is overall satisfactory. Somatic embryogenesis confirmed its effectiveness in eradicating phloem-limited viruses, probably because there is no translocation of these viruses from mother plant infected tissue to embryos via callus (which lacks vascular system) (4). The presence of eventual somaclonal variations and/or juvenility characters need to be carefully ascertained in regenerated plantlets after their transfer to the field: with this aim, an experimental vineyard will be planted in spring 2004.

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ELIMINATION OF *GRAPEVINE VIRUS A* BY CRYOPRESERVATION

Q. Wang[1,2], R. Gafny[2], P. Li[2], M. Mawassi[2], I. Sela[1] and E. Tanne[2]

[1]The Hebrew University, Faculty of Agricultural, Food and Enviromental Quality Sciences, Institute of Plant Sciences, Virus Laboratory, Rehovot 76100, Israel.
[2]ARO, The Volcani center, Department of Virology, Bet Dagan 20250, Israel.

Virus diseases have a major economical impact on viticulture. In many cases virus diseases cause a severe reduction of yield, and a reduction in the quality of the grapes, both in table grapes and in vine cultivars. In extreme cases, virus diseases bring about a severe decline and death of the affected plants.

The major mean of controlling grapevine virus diseases is the production of virus-free propagation material. Different methods have been used at various degrees of success. The most common ones are thermotherapy and meristem cultures, or a combination of both. Somatic embryogenesis has been suggested as well.

Cryopreservation has been introduced to plants since the 1960's (1, 2). Its main goal was to preserve genetic material with minimum variations. The method has been improved since to increase survival rate and regeneration. Cryopreservation has been carried out with shoot tips, meristems, cultured cells, calli and embryos. Cryopreservation was applied as a tool for virus elimination in a few cases (3, 4).

Grapevine virus A (GVA), closely associated with Kober stem grooving, is a component of the complex associated with the rugose wood syndrome (5). The virus was also detected in leafroll-diseased plants. This virus is difficult to eliminate by conventional methods such as heat therapy, meristem cultures and a combination of both. For this reason GVA was selected for the cryopreservation studies.

GVA-infected grapevines served as source material for in-vitro-established plantlets. Plants were maintained *in vitro* at 24^{o}C under a 16h photoperiod in a growth chamber. Shoot tips excised from these plants were cryopreserved using either the encapsulation-dehydration or the vitrification method.

In parallel, meristems (3 different sizes) were excised and were cultured in-vitro. Plantlets obtained either following cryopreservation or meristem culturing were transferred to a suitable medium , grown in-vitro and later established as plants under greenhouse conditions.

The presence of GVA in these plants was tested by western blotting with antibodies against the GVA movement protein (6). Plants were tested while still in culture and again 4 months after being established in the greenhouse.

This study demonstrates the elimination of GVA from infected grapevines (97%) compared to a mere 12% in meristem-regenerated plants. The size of the excised shoot-tip or meristem has no bearing on GVA elimination by cryopreservation, and adherence to very small meristems is not required.

We propose the use of cryopreservation as an efficient method for GVA elimination from infected grapevines.

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SOMATIC EMBRYOGENESIS, A RELIABLE METHOD FOR *GRAPEVINE FLECK VIRUS* FREE GRAPEVINE REGENERATION

C.F. Popescu,E.C. Buciumeanu and E. Visoiu

Research Station for Viticulture, Stefanesti, Arges, Romania

Ten years ago, *Grapevine fleck virus* (GFkV) was detected in same planting material for grapeyards. Since then, all cultivars from our farms were periodically checked by ELISA test in order to detect all infected plants and to eliminate them (2). In the same time we studied several aspects of fleck virus infection in different tissues-derived calli, and to establish the possibility of virus elimination through regeneration by somatic embryogenesis (1).

The causal agent of grapevine fleck disease was detected by ELISA tests in field grown plants, which were used as source for *in vitro* regeneration from ovules, anthers and leaves. During *in vitro* evolution, some genotipes (Mission, Coarna neagra and Ranai Magaraci) exhibited a significant response in callus induction from somatic tissues, relatively long-term embryogenic activity and high percentage of somatic embryos conversion into plants (3).

Initial ELISA tests were performed with calli in proliferation and maintenance phases. The readings in duplicate wells at 405 nm revealed that only a part of induced calli were infected with fleck particle. These data demonstrated that this virus, like other nepoviruses, is not restricted to anther-phloem tissues and it is transmitted in the new dedifferentiated cells. The migration of virus particles from one cell to another in calli, being an active process, depends on the degree of infection in the originally inoculated cells and on the length of time required for callus induction. This could be the explanation for the fluctuation of ELISA readings (positive and negative) during the growth and proliferation of the calli.

The embryos belonging to each cultivar and originated from one explant, exhibited a continuous variation of the optical density during *in vitro* culture, from uninfected (lower values than the negative control), to infected (two times higher values than the negative control). These results were correlated with the morphological status of the embryos: the samples from abnormal somatic embryos (cup-shaped, policothyledonary, without root primordia, chlorophyll deficiency and secondary embryos) were positive for virus infection, while the normal embryos were all free of virus particles. Also, they prove that the virus particles did not interfere with embryogenic ability and migrated to adjacent cells before the induction of embryogenic process. The high proportion of abnormal somatic embryos (53.3 % with Mission, 30.3 % with Coarna neagra and 25.7 % with Ranai Magaraci) and their low capacity of conversion into plants (27.3 %, 20.7 % and 10.3 % respectively) could be also the consequence of virus infection, inducing metabolic disturbances.

In opposition, with the same genotipes, the plants regenerated by direct embryogenesis from somatic tissues were free of any infection. In this case, it is supposed that the short time of culture necessary for direct regeneration was not enough for spreading the virus particles into the surrounding structures.

The regenerated plants obtained from the above mentioned structures with the three cultivars, Mission, Coarna neagra and Ranai Magaraci, were planted in the field and after three years all of them were healthy and exhibited normal growth and fruited.

The ELISA tests done in different stages of embryogenic process in order to detect fleck virus, indicated that only virus free tissues differentiated and produced normal plants. The results of this study showed that somatic embryogenesis induced in fleck infected structures, in the absence of heat treatment, could be a very efficient method of producing virus-free plants in grapevine.

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EFFECT OF ANTIVIRAL DRUGS IN *VITIS VINIFERA* INFECTED EXPLANTS

A. Panattoni and E. Triolo

Dipartimento di Coltivazione e Difesa delle Specie Legnose "G. Scaramuzzi", Università degli Studi, Via del Borghetto 80, 56124 Pisa, Italy

Viruses and virus-like diseases occur wordwide and have a detrimental impact on the vines and their crop.. Grapevine is known to host by the highest number of viruses so that ,sometimes, it is impossible to find healthy plants in the field. Currently, the only way to control viral infections is prevention, so the possibility of virus sanitation to obtain healthy germplasm for propagation appears as a mandatory step. Thermotherapy and meristem tip culture represent the most utilized techniques rather than chemotherapy, which is less used (3, 4).

In the course of surveys for grapevine (*Vitis vinifera* L.) sanitary selection in central Italy, we observed an exceedingly high level of infection by different viruses and theit combinations in cv. Sagrantino which prevented finding of healthy plants. Single infections were found in five selected vines: NM1, NM3 and MIL1 (Grapevine virus A, GVA), MF29 (*Grapevine leafroll-associated virus 1*, GLRaV-1) and AGO3 (*Grapevine leafroll-associated virus 3*, GLRaV-3).

The aim of this work was to test some antiviral drugs on the same host characterized by single virus infections, for identifying compounds that inhibits viral replication.

Internodes from naturally infected vines were collected and surface sterilized before transferring to culture tubes with fresh Murashige and Skooge medium (2). The explants were kept in a controlled environment chamber and transferred to proliferating medium at four weeks intervals. After an acclimation period, therapeutic drugs were added to the proliferating medium for different cycles depending on the behaviour of each virus. ELISA tests were conducted to assay the sanitary status of each explant before, during, and after each treatment. Selected antiviral drugs were: amantadine (1-adamantylamine hydrochloride $C_{10}H_{17}N$ HCl), DHT (2,4 dioxo-hexahydro-1,3,5-triazine $C_3H_5N_3O_2$). DHPA [(RS)-9-(2,3-dihydroxypropyl) adenine $C_8H_{11}N_5O_2$], ribavirine (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide $C_8H_{12}N_4O_5$) and the combination of DHPA/ribavirine, each at a not phytotoxic concentration (1).

At the and of chemotherapy, explants were maintained in *in-vitro* cultures for ELISA testing and only plantlets that were ELISA-negative were transferred to rooting medium, then transferred in *in-vivo* conditions to establish new sanitised plants.

According to ELISA results, amantadine and DHT were ineffective against all infections, while ribavirine always induced a significant virustatic effect and, in a small percentage of cases, a virucide effect , as shown by the difference of optical density observed between treated and non treated explants. DHPA itself gave a moderate virustatic effect, but combined with ribavirine seemed to increase the therapeutic effect.

These preliminary experiments confirmed the success of ribavirine, which proved to be a molecule effective against all viruses assayed, differently from the other coompounds which did not show any effect on virus replication.

These results require confirmation by molecular analysis, like RT-PCR and nested PCR amplification which is now underway, to definitely determine the health status of the treated explants

Table 1. Results of ELISA tests on *Vitis vinifera* cv Sagrantino after *in vitro* chemical treatments.

	Antiviral drugs				
	Amantadine	DHT	DHPA	Ribavirine	Rib+DHPA
	(40 μgml^{-1})	(40 μgml^{-1})	(40 μgml^{-1})	(20 μgml^{-1})	(20+40 μgml^{-1})
Virus					
GVA_{NM1}	Positive	Positive	Positive	Negative	Negative
GVA_{NM3}	Positive	Positive	Positive	Negative	Negative
GVA_{MIL1}	Positive	Positive	Positive	Negative	Negative
$GLRV_{1\ MF29}$	Positive	Positive	Positive	Negative	Negative
$GLTV_{3\ AGO3}$	Positive	Positive	Positive	Negative	Negative

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VIRUS-FREE GRAPEVINES MAY CONTRIBUTE TO SAFEGUARD AND EXPLOIT THE VITICULTURE IN THE DIFFICULT ENVIRONMENT OF THE 5 TERRE (NORTH-WEST ITALY)

F. Mannini[1], I. Gribaudo[1], D. Cuozzo[1], S. Tronfi[2] and F. Bonanini[3]

[1]Istituto Virologia Vegetale del CNR, Unità Staccata Viticoltura, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy
[2]Regione Liguria, Ispettorato Agricolo La Spezia, Via 24 Maggio 3, 19100 La Spezia, Italy
[3]Parco Nazionale delle 5 Terre, Via T. Signorini 118, 19017 Riomaggiore (SP), Italy

The 5 Terre area, located in Liguria (Italy), has environmental and cultural features of great value: among them, overhanging coasts over the Mediterranean Sea and about two thousand kilometres of dry-stone walls supporting the terraces where grapevine has been cultivated for centuries. These peculiarities have lead in 1999 to the creation of the 5 Terre National Park, which also includes some coastal wilderness and a marine protected area. Unfortunately, only part of the vineyards is still under cultivation: viticulture on the ancient terraces requires hard manual work and high costs while the low yield obtained from the old vines makes difficult to get profitable incomes from them. Nevertheless, keeping local viticulture alive means not only the safeguard of the traditional farming but also a support to local tourism, the protection of the steep hills from falling into the sea through the maintenance of dry stone walls, and the preservation of "a cultural site of outstanding value, representing the harmonious interaction between people and nature" (as said in the Justification for inscription of 5 Terre, Portovenere and three close islands in the UNESCO World Heritage List).

In this framework, a new vineyard has been planted in 2001. It is part of the activities of the 5 Terre National Park, in collaboration with the IVV-CNR, Grugliasco (TO). The vineyard is located in Riomaggiore (SP) and at the end of the project it will have an extension of 5 hectares, of which 4.5 have been already established so far. The vineyard results from the unification of about 120 small lots (formerly vineyards but abandoned and covered with wild Mediterranean bush) which the National Park rented out by owners. It has to be underlined that the total vineyard surface for the production of the VQPRD wine '5Terre' is less than 100 hectares, thus the surface involved in the project is important for this area. Before the vineyard planting, the terraces have been cleaned, restored and linked up, and a monorail track has been installed to allow a minimum mechanization.

The planted vines belong the three typical local varieties: Bosco, Albarola and Vermentino, from which the 5 Terre and the Sciacchetrà VQPRD wines are made. One of the reasons of unsatisfying performances of old 5 Terre vineyards is the strong diffusion of viral diseases (particularly phloem-limited viruses such as GLRaV-1 and 3, and GVA) (1). For Vermentino, the sanitary and genetic selection performed by IVV-CNR Grugliasco (formerly Centro Miglioramento Genetico e Biologia Vite – CNR) during the last 10 years made a number of naturally virus-free clones with good agronomical features available to grape-growers (2). For Bosco and Albarola it was not possible to find virus-free plants in the field, and the application of virus eradication procedures was necessary. Meristem culture has been applied since 1993 and several clones were produced which resulted negative to all the viral tests prescribed by certification protocol. These sanitated grape clones fulfil the requirements as for yield and grape quality (3; 4). Meristem culture has also been applied to Vermentino in order to increase the number of virus-free clones available for the cultivar.

In the new Riomaggiore vineyard a significant part of the plants used was from virus-free, selected clones. In addition, the present availability of numerous virus-free clones for each variety allowed to plant a policlonal vineyard thus safeguarding the genetic variability of the cultivars. All scions were grafted on certified 1103 Paulsen rootstock. Three years after the vineyard establishing and despite the difficult environment, the vines are growing vigorously and they have reached full production. The harvest will be conferred to the local Cooperative Winery, where most 5 Terre VQPRD wine is produced.

The described vineyard represents, to our knowledge, a rare example of broad-scale planting of sanitated grapevines. The healthy status of vines, added to the choice of locally-adapted cultivars, should ensure an optimal performance of the vineyard and, consequently, a good reward to grape-growers. This vineyard can also represent a permanent demonstration of good agronomical practices applied to the peculiar situation of a difficult environment with high landscape value.

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THE ROLE OF ROOTSTOCK GENOTYPE IN THE EFFECTS OF SINGLE AND MIXED INFECTIONS OF GRAPEVINE VIRUSES

D.A. Golino, S. Sim and A. Rowhani

Department of Plant Pathology, University of California, Davis, California, U.S.A. 95616

Newly replanted grape (*Vitis vinifera*) vineyards in California in the 1990s were observed failing with disease symptoms characteristic of virus infection. This epidemic occurred during a planting cycle which involved a dramatic change in rootstock genotypes. Disease was associated with vineyards using certified rootstock, field grafted with scion buds from apparently healthy commercial vineyards. We hypothesized that virus infections which were latent in vines growing on their own roots or grafted to V. hybrid 'AXR-1' were causing severe disease on the alternative rootstocks which were coming into common use. This phenomena has been widely referred to as the latent virus problem in California (2). Since diagnosis of grapevine virus infections can be complex, grapevine accessions were taken from vineyards showing typical symptoms of the phenomena and propagated into a permanent collection. These accessions have been analyzed for infection with grapevine viruses using traditional woody and herbaceous indexing, ELISA tests, and RT-PCR tests. In addition, all accessions were indexed on the rootstock 'Freedom' which was associated with severe latent virus problems early in the epidemic. Diagnosis was completed over a number of years.

A cause and effect relationship has been confirmed between the presence of certain viruses in propagating stock and the decline of young vines. A total of 36 virus accessions have been indexed on Freedom. In 18 cases, severe latent virus effects on Freedom rootstock were observed. In all but one case, when 'Freedom" was severely affected, both GLRaV-2 and GVB are present in the virus accession. In the case of that single exception, the vitiviruses GVA and GVC were both present may have contributing the same disease factor as GVB (3, 4). Conversely, all of the virus accessions that did not cause a severe reaction on Freedom tested negative for GLRaV-2 + GVB.

We have demonstrated that the combination of GLRaV-2 and GVB described above leads to severe stunting on Freedom rootstock. We named this phenomena virus-induced rootstock decline (VIRD). VIRD is highly correlated with the presence of both GLRaV-2 and GVB, which may be acting synergistically. It is important to note that this effect is independent of any rootstock/scion relationship or incompatibility as has been observed in other disease cases. Although rootstock/scion interaction may be involved in this and other virus disease syndromes, in this case, severe effects of virus can be noted on the rootstock alone.

Tests were conducted to compare the relative resistance of popular grapevine rootstocks to both single viruses and mixed infections which caused VIRD on 'Freedom'. Selected virus sources were grafted to each of 22 grape varieties (largely rootstock cultivars) including 101-14 Mgt, 110R, 1103P, Kober 5BB, 3309C, 140Ru, Teleki 5C, 420A Mgt, Ramsey, Riperia Gloire, 1613C, 1616C, Schwarzmann, SO4, 44-53 Malague, ,Dog Ridge, AXR, St. George, Chardonnay, Cabernet Franc, Freedom and Harmony. 40 plants were budded per treatment per cultivar and planted in 5 randomized blocks with 8 plants/block for a total of 240 plants/ variety and 5,280 total plants. Virus treatments included: non-grafted control, grafted healthy control, LR 101 (GLRV-3), LR 102 (GLRaV-1, GLRV-2, GVB), LR 109 (GFkV, GLRaV-2, GLRaV-3, GVC), and CB 100 (GLRaV-2,GVB). These are all established virus sources that have been extensively indexed by multiple techniques (1). LR 102, LR 109, and CB 100 have been demonstrated to cause severe VIRD.

Graft inoculations were accomplished by chip budding two dormant buds from virus-infected source plants into green growing rootstocks produced in our greenhouses. The inoculation buds were allowed to callus and take for several weeks before planting. About 2 months after inoculation, bud take was observed (dead buds may mean that virus transmission has not been achieved), and the inoculum buds were removed. Plants for which neither inoculum bud healed were removed from the data analysis. Data taken on these blocks over the next 2 years included: survival, symptoms, trunk diameter, length of the longest shoot in early spring, and dormant pruning weight. Grafts were observed for necrosis and abnormalities. Data is now available for all of the cultivars above except 44-53 Malague and Dog Ridge which will be complete in the spring of 2005.

AXR was clearly unaffected by any of the inoculated viruses (Fig 1). There was no significant difference between healthy and virus-inoculated plants in 2 of 3 years data of length of the longest shoot nor in 2 years of pruning weight data. Shoot length in year 2 ranged from 90 to 100% of healthy; pruning weight in year 2 ranged from 89 to 121% of healthy. Survival was 100% over all 3 years of the study. Selected plants were tested by ELISA and PCR and did test positive for virus, providing evidence that AXR, while infected with virus, did not suffer any detectable effects of the virus.

Shoot length in St. George and Chardonnay was not significantly affected although some differences in growth were observed (Fig. 1). Pruning weight in St. George and Chardonnay inoculated with LR109 was significantly reduced to 56% and 65% of healthy, respectively (data not shown). Freedom and Harmony were severely and significantly affected by CB100, LR102 and LR109, all multiple infections. In Freedom by year 2, both spring shoot length and dormant pruning weight ranged from 2 to 25% of healthy for these three treatments. In Harmony both parameters ranged from 14 to 49% for the same virus treatments (Fig.1). Percent survival was high over 3 years, 100% in many treatments, even though the plants were severely stunted.

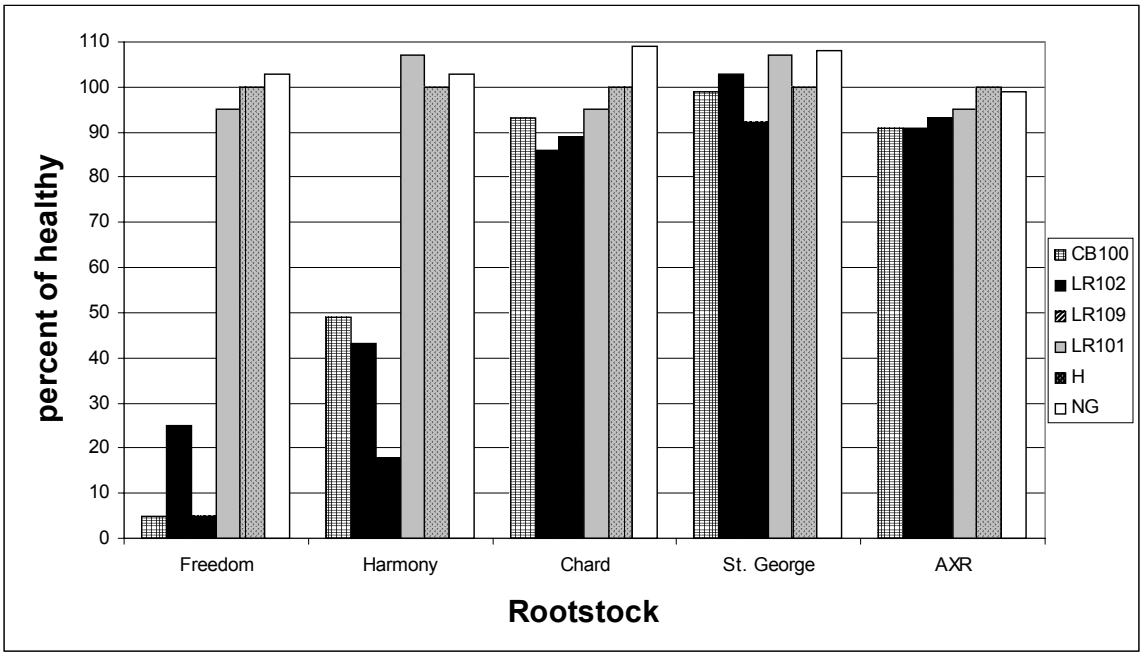
Kober 5BB and SO4 had a less severe response to infection. In Kober 5BB, only one virus accession, LR102, significantly reduced shoot length to 85% of healthy; pruning weight was 66% of healthy. In SO4, two virus treatments significantly reduced shoot length to 59% and 89% of healthy; pruning weight was 57% and 55 % of healthy for CB100 and LR102 respectively.

In 1613C, Schwarzmann, and 420A the first year measurement of shoot length showed significant stunting by the multiple virus infection treatments, the worst cases being 18% (CB100), 31% (LR102), and 39% (CB100) of healthy for the respective rootstocks. It would be expected that these effects will be even more severe in year two.

This data provides clear support for our original hypothesis that the reason virus problems became more evident when growers changed from AXR to new rootstocks was because scions grafted to AXR often can be symptomless carriers of viruses. When scion wood was taken from a vineyard that had been doing well on AXR and grafted to a different rootstock, virus disease caused decline in growth and productivity.

It also provides supporting evidence for our hypotheses that rootstock response to virus infection depends on the rootstock genotype and the virus type. Rootstocks differ widely in virus susceptibility and rootstock growth and survival is affected by virus infection. No graft union is necessary for detrimental virus effects to appear, although graft union symptoms may be a symptom of rootstock decline.

Figure 1. Average length of longest shoots in spring, 2 years after inoculation with selected virus isolates, expressed as a percent of healthy; H = healthy control, NG = not grafted control.



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FLURPRIMIDOL AND D-MANNITOL AS TOOLS FOR *IN VITRO* STORAGE OF GRAPEVINE GERMPLASM

I.Tsvetkov[2], N.Ioannou[1], A.Hadjinicoli[1], A.Hadjinicolis[1] and A.Atanassov[2]

[1]Agricultural Research Institute, P.O. Box 22016, 1516 Nicosia, Cyprus, e-mail: ioannou@arinet.ari.gov.cy
[2]AgroBioInstitute, 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria, e-mail: ivantsvetkov@hotmail.com

Introduction

In vitro storage under growth retarding conditions delays the necessity for frequent transfers to fresh media and allows flexibility in meeting market demand, leading to efficient management of labour (4). For many vegetatively propagated species, preservation techniques are sufficiently advanced to envisage their immediate utilization for large-scale experimentation in genebanks (2). Two approaches are taken to *in vitro* storage: (a) slow growth and (b) cryopreservation. Slow growth is achieved by maintaining cultures (usually shoots/plantlets) at a reduced temperature or in the presence of osmotic or hormonal inhibitors (6). The most commonly employed protective substances are dimethylsulfoxide (DMSO), mannitol, sorbitol, sucrose and polyethyleneglycol (PEG). They have principally an osmotic action but some of them (e.g. DMSO) can enter cells and protect cellular integrity during eventual use of cryopreservation (5). Ancymidol, abscisic acid (ABA), paclobutrazol and flurprimidol are most often reported to be useful for *in vitro* storage as growth inhibitors and retardants (1, 3, 7). The aim of the present work was to study the potential use of flurprimidol and mannitol for medium-term *in vitro* storage of grapevine genebank.

Materials and methods

Twenty two different grapevine varieties from the Agricultural Research Institute (ARI) *in vitro* grapevine collection were used as source of plant material. Murashige & Skoog growth medium including Nitsch vitamins, supplemented with 0.3 mg/l IBA, 100 mg/l gluthatione, 200 mg/l L-pheniylalanine, 200 mg/l L-glutamine, 250 mg/l casein hydrolysate, 250 mg/l malt extract and 2% sucrose was used. The medium was solidified with 0.6 % Micro agar and pH was adjusted to 5.9. The experimental variants were additionally supplemented with 1mg/l flurprimidol (treatment A) or with 4% D- mannitol (treatment B), while the control (C) consisted of the above described medium without growth inhibitors. Explants were cultured in 20x200 mm glass tubes, each containing 10 ml medium and exposed to 16/8 hours photoperiod and 25^0C temperature. Results were observed after 40 days in culture.

Results and disscussion

In general, a remarkable decrease of the plant growth was observed in both experimental variants A and B. Such decrease was expressed as reduction of the length of both new shoots and roots, as well as in fresh weight (Table 1). Fifteen of the screened varieties (68%) did not develop any roots in variant B, which could reflect negatively on the new plants later. Storage conditions should preserve both the post-storage quality and re-growth potential. We recommended avoiding the use of D-mannitol or using lower quantity (2% up to 2.5%) in future experiments. Variant A (with 1mg/l flurprimidol) appeared to be more balanced, enabling both *in vitro* storage and preservation of plant re-growing potential. The present data indicate the potential usefulness of the growth retardant flurprimidol for *in vitro* conservation of grapevine germplasm.

Table 1. Results of grapevine *in vitro* germplasm conservation experiments

Variety	Shoots (cm)			Roots (cm)			Fresh weight (g)		
	A	B	C	A	B	C	A	B	C
Flame seedless	3.0	3.5	3.5	3.0	1.0	4.0	0.15	0.10	0.18
Merlot	3.0	2.0	4.0	1.5	-	0.5	0.11	0.02	0.14
Muscat Ottonel	2.5	3.0	3.5	-	-	4.0	0.05	0.04	0.34
Shiraz	2.0	2.0	3.0	1.0	-	1.0	0.10	0.03	0.26
Sylvaner	2.5	2.0	4.0	-	-	3.0	0.03	0.03	0.09
Allicante Bouchet	1.0	1.0	7.0	0.5	-	3.5	0.20	0.04	0.54
Calmeria	1.5	2.5	5.5	1.0	-	4.5	0.32	0.04	0.25
Malvasia Blanka	2.5	2.0	6.0	1.0	-	4.0	0.10	0.02	0.21
Thompson Seedless	2.0	2.0	5.0	3.0	-	5.0	0.12	0.05	0.20
Fantasy Seedless	2.0	2.0	6.0	-	1.0	4.0	0.05	0.14	0.37
Saint Emillion	3.5	2.0	7.0	0.5	0.5	4.0	0.13	0.20	0.40
Grenache	4.0	3.5	6.0	1.0	0.5	3.5	0.12	0.07	0.64
Emerald Seedless	3.0	2.0	7.0	1.0	-	3.5	0.15	0.08	0.25
Cabernet Sauvignon	3.5	4.0	4.5	1.0	-	5.0	0.04	0.04	0.40
Gamay	1.5	2.5	4.0	1.0	1.5	1.5	0.07	0.07	0.07
Pinot Noir	3.0	3.0	4.0	1.0	-	2.5	0.29	0.10	0.14
Cabernet Franc	3.0	1.5	5.0	2.0	-	3.0	0.21	0.05	0.26
Prima	2.0	3.0	5.0	1.0	0.5	4.0	0.56	0.21	0.39
Crimson Seedless	4.0	1.5	6.0	2.5	-	2.0	0.60	0.09	0.37
Autumn Royal	3.0	3.0	5.5	0.5	-	3.0	0.10	0.08	0.29
Cardinal	2.0	2.5	4.0	1.0	-	2.0	0.09	0.06	0.14
Queen	2.0	3.0	4.5	1.0	0.5	2.5	0.10	0.06	0.26
Mean	**2.57**	**2.43**	**4.72**	**1.11**	**0.25**	**3.18**	**0.17**	**0.07**	**0.28**

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EFFECT OF GRAPE VIRUSES (FANLEAF, YELLOW MOSAIC, LEAFROLL) ON QUANTITY AND QUALITY OF YIELD AND ON THE STATUS OF GRAPEVINE PLANTATION

A. Simon, L. Bodor and G. Bujtás

Plant and Soil Protection Service of county Bács-Kiskun, Kecskemét, Hungary

In the grape growing countries of the world, approx. 30 grapevine viruses, virus-like diseases and phytoplasms have been so far described, and 15 virus of them could be detected in Hungary. Among these diseases, fanleaf and leafroll cause damages of economic importance. Virus diseases of grape cause gradual wilting of the plants, qualitative and quantitative yield losses as well as shorter productive period.

It was decided to start provocative experiments under Hungarian conditions because, up to now, only foreign data have been available to prove the facts mentioned above. A 1 hectare large plantation was established in 1982 with virus-free propagation material on disinfected soil. The varieties used in the experiment: Chardonnay, Ezerfürtű, Pinot noir.

System of plantation and mode of cultivation: 350 x 120 cm of spacing between rows and plants using high cordon cultivation.

Artificial inoculation was made by using the method of chip-transmission in 1984-85 on half the plants in each row.

Number of replicates: 4, randomized.

The infected materials (fanleaf combined with yellow mosaic and leafroll viruses) were taken from virus infected plants designated in bearing vineyard and from collection of pathogen isolates. The white grapevine varieties (Chardonnay and Ezerfürtű) were inoculated by *Grapevine fanleaf virus*, which was mixed with *Yellow mosaic virus* and as it appeared later with *Grapevine yellow phytoplasma*. The red variety, Pinot noir, was inoculated by *Grapevine leafroll-associated virus 3*.

No cross infection has been observed between the infected and healthy plants close to each other during the visual inspections, confirmed by ELISA tests.

The virus symptoms were well visible even one year after inoculation. The most spectacular changes were observed in the yield losses. At an average of 18 years, some 51% of yield reduction appeared on the infected plants. Similar changes were produced with the average weights of berries and clusters. Great differences were also observed in the impact on endurance of climatic factors, like winter frost. At the harvest the whole quantity of grapevine, 100 cluster and 100 berries of each treatment, (e.g. Chardonnay , inoculated) was weighed, and the sugar content was determined at every treatment. After harvest vinification experiments were carried out, the wine of each treatment was assessed.

These studies in experimental vineyard inoculated with the two economically most important and dangerous grapevine viruses demonstrated the fact, that even in Hungary these casual agents can dramatically decrease the quantity and the quality of grapevine and of wine as well.

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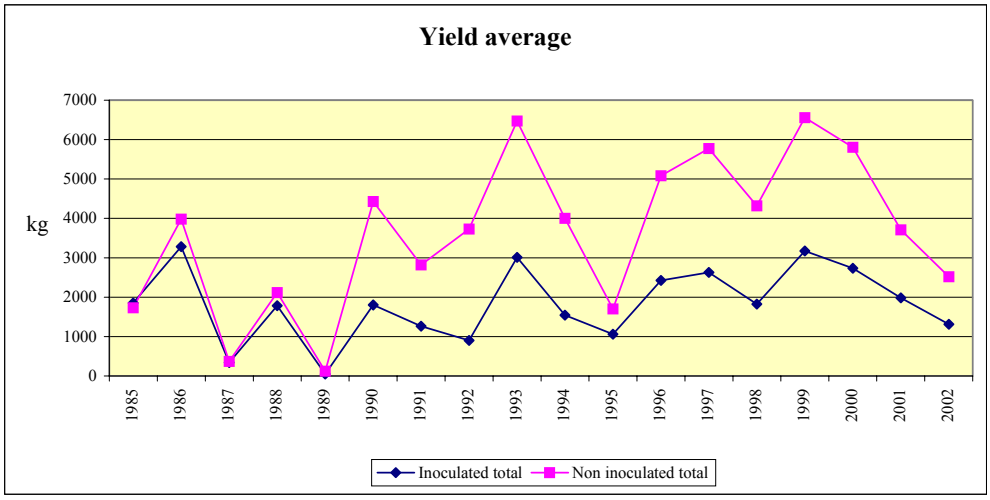
Yield quantity of grapevine in the experimental vineyard

TREATMENT/YEAR (kg)	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	Total
Ezerfürtű inoculated	510	1641	0	30	4	238	239	139	560	225	199	648	684	556	1203	552	718	311	8457
Ezerfürtű non inoculated	639	2058	0	192	15	1260	491	1338	1840	942	785	1994	1644	1686	2484	1652	1210	828	21058
Ezerfürtű total	1149	3699	0	222	19	1498	730	1477	2400	1167	984	2642	2328	2242	3687	2204	1928	1139	29515
Chardonnay inoculated	186	858	115	343	12	564	293	233	1026	488	88	571	530	486	683	693	542	658	8369
Chardonnay non inoculated	124	1043	186	554	70	1559	959	1246	2188	1444	156	1122	1483	990	1726	1379	1082	1111	18422
Chardonnay total	310	1901	301	897	82	2123	1252	1479	3214	1932	244	1693	2013	1476	2409	2072	1624	1769	26791
Pinot noir inoculated	1155	787	226	1411	32	1002	732	534	1423	833	774	1205	1411	784	1286	1488	721	344	16148
Pinot noir non inoculated	965	879	186	1371	37	1611	1370	1146	2444	1615	762	1963	2645	1639	2344	2776	1421	581	25755
Pinot noir total	2120	1666	412	2782	69	2613	2102	1680	3867	2448	1536	3168	4056	2423	3630	4264	2142	925	41903
Inoculated total	1851	3286	341	1784	48	1804	1264	906	3009	1546	1061	2424	2625	1826	3172	2733	1981	1313	6027
Non inoculated total	1728	3980	372	2117	122	4430	2820	3730	6472	4001	1703	5079	5772	4315	6554	5807	3713	2520	12040
Total yield quantity	3579	7266	713	3901	170	6234	4084	4636	9481	5547	2764	7503	8397	6141	9726	8540	5694	3833	98209
Yield of inoc. expressed in % of healthy	107	83	92	84	39	41	45	24	46	39	62	48	45	42	48	47	53	52	55



CHANGES IN AGRONOMICAL AND OENOLOGICAL PERFORMANCES OF CLONES OF THE GRAPEVINE CV GEWÜRZTRAMINER AFTER *GRAPEVINE FANLEAF VIRUS* ELIMINATION BY HEAT THERAPY

U. Malossini, A.M. Ciccotti, P. Bragagna, M.E. Vindimian, S. Moser, G. Versini and G. Nicolini

Istituto Agrario di San Michele all' Adige (IASMA), 38010 San Michele all'Adige, Italy

Grapevine fanleaf virus (GFLV) is the most widespread nepovirus involved in the grapevine degeneration. It is generally reported that GLFV elimination induces a strong modification of vine behaviour, an increase of cane length and leaf surface, a dramatic increase of vigour and yield. In a warm climate the increase of vigour and yield of GFLV-free materials did not penalize the berry juice composition: more doubtful are the results obtained in a cool climate area (4). The genetic variability within a cultivar is the basis of clonal distinction, but the virus infections may have great practical implication on the studied phenotypical variability. The clonal selection carried out at IASMA confirmed interesting differences, as for agronomical performances and analytical and sensorial profiles of wines, among Gewürztraminer's clones (3). Only a few clones (both healthy and affected by virus) showed always varietal typicality. It was confirmed that the profile of bound forms of certain aroma compounds (e.g. geraniol and linalool) well discriminated the *aromatic* and *neutral* genotypes (3). In this preliminary work was investigate the influence of GFLV on the characteristics of two infected clones of grapevine cv Gewürztraminer in comparison to the same clones after sanitation.

The present study deals with the differences in agronomical and oenological performances between GFLV-infected (MP) and heat-treated healthy (HT) progenies of two Gewürztraminer's clones. In 1997, the originally GFLV-infected clones 920 and 921 (respectively as *aromatic* and *neutral* Gewürztraminer's genotypes) were heat-treated according to in vitro thermo-therapy (1). Established daughter vines of both original (MP) and heat treated (HT) clones were tested by ELISA for GFLV, ArMV, GLRaV-1, GLRaV-3 and GVA using commercial kits (Agritest, Valenzano-Bari, Italy). ELISA tests were carried out on leaves and woody samples from each original (MP) and heat-treated (HT) vines in five years (1998-2002). HT progeny of 920 and 921 clones resulted GLFV-free. MP and HT both *ex-vitro* material was green-grafted on virus-certified rootstocks (Kober 5BB). An experimental vineyard in sandy soil was established in the year 2000 at plane (210 m a.s.l., San Michele a/A - Italy). More of 10 plants for each trial are Guyot-system trained. In June 2001 (only for 920 clone), sample of 10 leaves with 4 different ages (node position on shoot) from both MP and HT vine were collected. The length of shoots, blades (main vein) and leaf-petioles were measured. Amounts of total chlorophyll (Chl) were spectrophotometrically (2) and by chlorophyll-meter Minolta SPAD-502 (5) determined. All measurements of Chl and fluorescence were performed, on detached leaves, with portable PAM-2000 fluorimeter (Walz, Effeltrich, Germany). In 2002 instead, were repeated the measures of SPAD index on collected leaves during the veraison (31 July) and at harvest (9 Sept.) from MP and HT plants of both clones (920 and 921). Additionally, the fresh weight of blades and petioles were measured and chemical analysis of these were carried out (5). At the harvest (2002) grape yield and must composition (Brix degrees, total acidity and pH) after grape crushing, short skin-contact, pectolitic enzyme addition and overnight cool-settling of the juice were recorded for each plot. Free and bound (as aglycons) monoterpene aroma compounds have been quantified in the juice after frozen storage according to Versini et al. (7) by HRGC-MS. Some 2002 data were processed by ANOVA with statistical software (6).

In 2001 vintage, only for clone 920 the sanitation showed an increase of shoots (Fig.1) and veins length (Fig. 2), but a decrease of total chlorophyll contents (Fig. 3) and SPAD values (Fig. 4). Moreover in 2002 all the considered sources of variation have a statistical significance on the leaves data (Table 1). Data of MP and HT leaves of the two clones 920 and 921 confirmed the increase of blade and petiole size (as for weight and length) in healthy vines. Lower content of nitrogen, phosphorus and potassium and a significantly greater level of calcium, magnesium and manganese have been found in HT leaves (Tab. 2). As for aroma compounds (Tables 4a,4b), 920 clone is confirmed as aroma-rich one for both forms (under them, geraniol and rose oxide are the most aroma variety contributors), while the 921 as a middle-low aroma clone. The HT-plants are in the first case richer than the MP vines, on the contrary for the 921 clone. This fact usually could be attributed to a different ripening stage of the grape, but in this case both HT-plants showed the higher sugar and a lower acidity level.

Regardless to the sanitary level, some genotypic important clonal aspects linked to oenological goals as aroma profile, pH and total acidity are maintained. Virus elimination surely improves the plant status and some agronomical characters.

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Fig.1: shoot's lenght of Gewürztraminer's grapevine (clone 920 - the 25th June 2001)

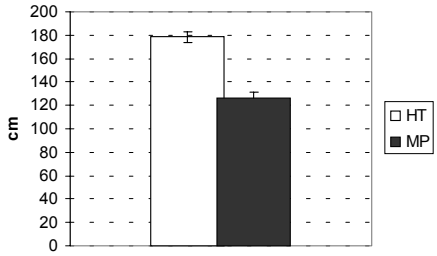

Fig.2: lenght of main vein on detached leaves by different age (clone 920 - the 25th June 2001)
[node position 1= basal]

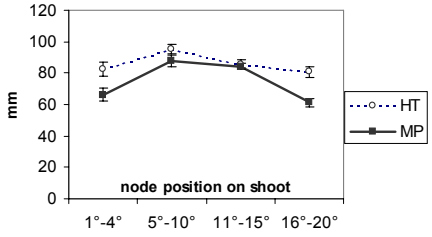

Fig. 3: total chlorophyll of detached leaves by different age (clone 920 - the 25th June 2001)
[node position 1= basal]

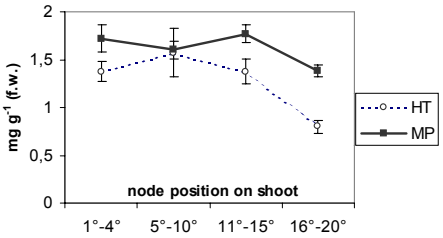

Fig. 4: SPAD values of detached leaves by different age (clone 920 - the 25th June 2001)
[node position 1= basal]

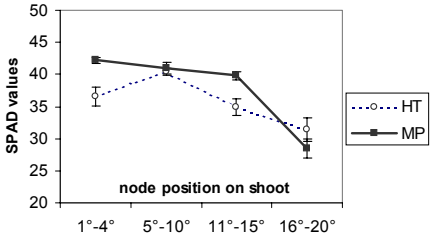

Table 1. Values of F and their statistic significance from ANOVA relatively to leaves (blades and petioles) of two GFLV-infected Gewurztraminer's clones, in relief in healty condition and date [year 2002].

Characteristics - / - Source of variation	Sanitation	Clone	Date
d.f.	1	1	1
leaf's weight (g)	9,1 *	0,1 ns	25,2 **
petiol's weight (g)	13,2 *	0,2 ns	7,4 *
blade's weight (g)	7,8 *	0,1 ns	31,1 **
vein's length (mm)	20,9 **	6,6 ns	9,9 *
petiol's length (mm)	61,1 **	16,9 **	24,0 **
SPAD index	2,0 ns	248,0 **	27,6 **
N blade (% s.s.)	17,2 *	4,5 ns	128,9 **
P blade (% s.s.)	7,2 ns	3,2 ns	7,2 ns
K blade (% s.s.)	149,4 **	10,8 *	17,4 *
Ca blade (% s.s.)	26,3 **	4,5 ns	4,5 ns
Mg blade (% s.s.)	30,2 **	0,6 ns	7,5 *
Mn blade (% s.s.)	93,7 **	11,9 *	5,8 ns
Bo blade (% s.s.)	9,0 *	289,0 **	25,0 **
N petiol (% s.s.)	3,0 ns	0,6 ns	5,5 ns
P petiol (% s.s.)	51,6 **	37,3 **	62,5 **
K petiol (% s.s.)	34,3 **	1,2 ns	45,2 **
Ca petiol (% s.s.)	12,8 *	22,0 **	11,1 *
Mg petiol (% s.s.)	12,9 *	6,1 ns	19,0 *
Mn petiol (% s.s.)	34,0 *	12,1 *	11,2 *

Note: (n.s.) not significance (*) significance between 95 and 99% (**) significance of 99% or superior

Table 2: Means of any characteristics of MP and HT leaves (blade and petioles) from two Gewürztraminer's clones, in relief of 2 date (see Tab. 1).

	MP	HT
leaf's weight (g)	2,41 b	3,10 a
petiol's weight (g)	0,61 b	0,80 a
blade's weight (g)	1,80 b	2,31 a
vein's length (mm)	81,8 b	89,8 a
petiol's length (mm)	69,5 b	84,3 a
SPAD index	41,1	41,4
N blade (% s.s.)	2,39 a	2,08 b
P blade (% s.s.)	0,26	0,25
K blade (% s.s.)	1,20 a	0,99 b
Ca blade (% s.s.)	3,15 b	3,48 a
Mg blade (% s.s.)	0,28 b	0,35 a
Mn blade (% s.s.)	101,8 b	139,0 a
Bo blade (% s.s.)	31,8 b	32,5 a
N petiol (% s.s.)	0,56	0,54
P petiol (% s.s.)	0,55 a	0,45 b
K petiol (% s.s.)	2,77 a	2,02 b
Ca petiol (% s.s.)	2,19 b	2,41 a
Mg petiol (% s.s.)	0,80 b	0,94 a
Mn petiol (% s.s.)	25,8 b	48,0 a

Note: different letters of the same variable given significantly differ (p 0.05) by Tukey's test

Table 3: Means of any characteristics of grape of two Gewürztraminer's clones (MP vs HT). Vintage 2002

	MP	HT
grape yield (kg)	0,184	0,550
weight cluster (g)	34,7 b	59,6 a
Brix degrees	20,15	21,00
total acidity (g/L)	5,70	6,34
pH	3,45	3,36
% stem / cluster (% g/g)	16,3 a	8,1 b

Note: different letters of the same variable given significantly differ (p 0.05) by Tukey's test

Tab. 4a. Aroma compounds of juice (vintage 2002) of Gewurztraminer's clones in free form (µg/L n-heptanol).

	920 HT	920 MP	921 HT	921 MP
MONOTERPENES				
trans furan linalool oxide	0,4	0,2	<0.1	0,2
cis furan linalool oxide	0,5	0,6	0,2	0,2
trans pyran linalool oxide	9,3	6,6	0,6	1,3
cis pyran linalool oxide	2,1	1,3	0,3	0,4
linalool	1,1	0,9	0,2	0,3
α–terpineol	0,9	0,7	0,6	0,4
citronellol	7,4	3,6	0,2	0,3
nerol	90,0	42,5	1,0	4,3
geraniol	260,0	137,5	3,3	16,5
trans geranic acid	31,0	13,0	0,6	1,8
Ho-diendiol (I)	35,0	17,0	4,0	3,5
cis 2,6-dimethyl-2,7-octadiene-1,6-diol	38,5	21,5	1,4	5,0
trans rose oxide	0,60	0,20	<0.01	0,04
cis rose oxide	0,20	0,07	<0.01	<0.01

Tab. 4b. Aroma compounds of juice (vintage 2002) of Gewurztraminer's clones in bound form (µg/L n-heptanol).

	920 HT	920 MP	921 HT	921 MP
MONOTERPENES				
trans furan linalool oxide	27,0	17,0	2,0	4,6
cis furan linalool oxide	24,0	12,0	2,9	3,7
trans pyran linalool oxide	12,0	6,6	1,0	1,5
cis pyran linalool oxide	7,5	2,8	0,9	1,2
linalool	13,5	5,2	0,4	1,2
α–terpineol	22,0	12,5	5,8	5,4
citronellol	3,5	18,0	0,6	2,1
nerol	297,0	130,0	3,9	19,5
geraniol	782,5	525,0	27,0	73,5
trans geranic acid	125,0	55,0	1,4	8,4
Ho-diendiol (I)	56,0	27,0	3,1	6,7
cis 2,6-dimethyl-2,7-octadiene-1,6-diol	121,0	91,5	13,5	31,0
trans rose oxide (*)	2,50	1,30	<0.01	0,04
cis rose oxide (*)	0,90	0,15	<0.01	0,02

(*) from an aglycon precursor

GRAPEVINE VIRUSES (GLRAV-1+GVA) INHIBITS PIGMENTS, RUBPC AND PHOTOSYNTHETIC ACTIVITIES IN FIELD GROWN GRAPEVINE (*VITIS VINIFERA* L.CV. MARZEMINO) LEAVES

U. Malossini, I. Roncador, A.M. Ciccotti, M. Bertamini and N. Nedunchezhian

Istituto Agrario di San Michele all'Adige, 38010 San Michele all'Adige, Italy

The most common methods to eradicate viruses are thermotherapy *in vivo*, *in vitro* and meristem culture (3). The present work was to investigate the effect of GLRaV-1 and GVA infection mainly on pigments, soluble proteins, ribulose-1,5-bisphosphate carboxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Marzemino) leaves.

The present study deals with virus elimination in grapevine using *in vitro* thermotherapy. One node-segments of a (GLRaV-1 and GVA) infected clone of 'Marzemino' were surface sterilized, positioned in solidified culture medium and kept in a thermotherapy chamber provided by cool white fluorescent lamps (40 μE m^{-2} s^{-1}) with 16/8 h photoperiod at 34°C for 40 days. After this treatment the axillary bud from each green shoot explant was aseptically excised and subcultured on same medium supplemented with 2 mg/l of BA (5). Established daughter vines of original (MP) and heat treated (HT) clone were tested by ELISA for GLFV, ArMV, GLRaV-1, GLRaV-3 and GVA, using a commercial kit (Agritest, Valenzano-Bari, Italy). ELISA tests were carried out on leaves and dormant canes samples of both (MP) and (HT) vines for five years (1998-2002). HT vines resulted GLRaV-1 and GVA free. Both original and heat-treated *ex-vitro* material was propagated on virus-certified rootstocks (Kober 5BB). An experimental vineyard with two blocks was established in the year 2000 at Ala (South Trentino, Italy). The vines are simple pergola system trained. In 2002, samples of 20 leaves from MP and HT plants were gathered at veraison (5 Aug.) and at harvest (16 Sept.). Fresh weight of blades and petioles and length of veins and petioles were measured. Chemical analysis of blades and petioles were carried out (9). Amounts of chlorophyll (Chl), carotenoids (Car) and total soluble proteins were spectrophotometrically determined by two methods (7) (2). The crude leaf extract isolated and assay of ribulose-1,5-bisphosphate carboxylase (RuBPC) activity was measured by Bowes and Ogren (1). All measurements of Chl fluorescence were performed, on detached leaves, with portable PAM-2000 fluorometer (Walz, Effeltrich, Germany). Thylakoid membranes were isolated and reactions of photosynthetic electron transport mediated by whole chain (H_2O → MV), PSII (H_2O → DCBQ, H_2O → SiMo) and PSI ($DCPIPH_2$ → MV) were measured as described by Nedunchezhian *et al*. (8). Thylakoid membranes and crude leaf extracts were separated using the polyacrylamide gel system of Laemmli (6).

The contents of Chl and Car were decreased in MP leaves (Fig. 1). This would be due to the grapevine virus, witch probably enhanced the chlorophyllase activity in the leaves. In MP leaves the Car/Chl ratio increased while the Chl *a/b* ratio decrease (Fig. 2). Total soluble proteins and RuBPC activity were reduced markedly in MP leaves (Fig. 3). This relatively low level of soluble proteins might have been due to decrease in the synthesis of RuBPC, the major soluble protein of leaf (Fig. 3). Such reduction in the RuBPC was found due to inhibition of protein synthesis induced by grapevine virus in leaves. This is also supported by SDS-PAGE analysis of crude leaf extracts of RuBPC proteins showed a marginal loss of both large (55 kDa) and small (15 kDa) unit proteins in MP leaves (Fig. 4b). The HT leaves showed a good PSII activity, measured as the Fv/Fm and the Fv/Fm was decreased in MP leaves. The decrease in Fv/Fm ratio was mainly due to decrease in variable fluorescence (Fig. 5). Analysis of various electron transport chain, the PSII and whole chain electron transport markedly inhibited by virus (Fig. 6). Among the various electron donors, DPC and NH_2OH were found to be slightly restoring the PSII activity in MP leaves (Fig. 7). Supporting evidence for the damage to PSII activity was obtained from the thylakoid polypeptide analysis: a comparison of thylakoids of MP leaves with those of the HT leaves showed specific loss of 33, 27-25, 23 and 17 kDa polypeptides (Fig. 4a). Analysis of various mineral contents, not significant changes were observed between HT and MP leaves (Table).

In this work we have studied the influence of grapevine virus (GLRaV-1 + GVA) on some features of the thylakoids from field grown grapevine (cv. Marzemino) leaves. Total chlorophyll, carotenoids, soluble proteins and ribulose-1,5-bisphosphate carboxylase level decreased in virus infected (MP) leaves. In isolated thylakoids, grapevine virus (GLRaV-1+GVA) caused marked inhibition of whole chain and photosystem (PS) II activity. The artificial exogenous electron donors, DPC and NH_2OH marginally restored the PSII activity in MP leaves. The Fv/Fm was also decreased in MP leaves without increasing Fo. The marked loss of PSII activity in MP leaves was evidently due to the loss of 33, 27-25, 23 and 17 kDa polypeptides. The content of various minerals are slightly changed in MP leaves. Thus, our results suggest that the loss of pigments, soluble proteins, RuBPC and photosynthetic activities in infected leaves was due to the virus induced rapid senescence or aging in field grown grapevine leaves.

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Fig. 1: contents of Chl and Car in leave

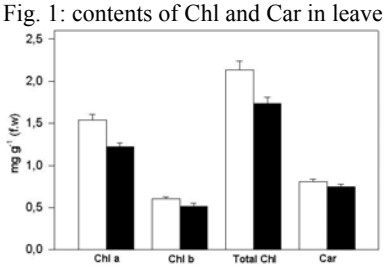

Fig. 2: Chl *a/b* and Car/Chl ratio in leaves

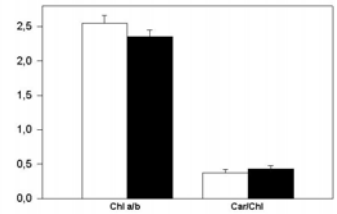

Fig. 3: total soluble protein and RuBPC activity in leaves

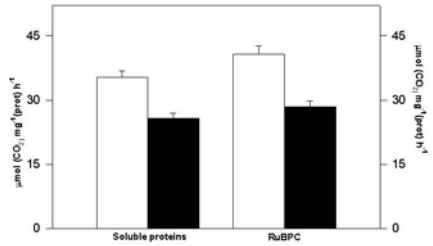

Fig. 4: SDS-PAGE analysis of crude leaf extracts of RuBPC proteins

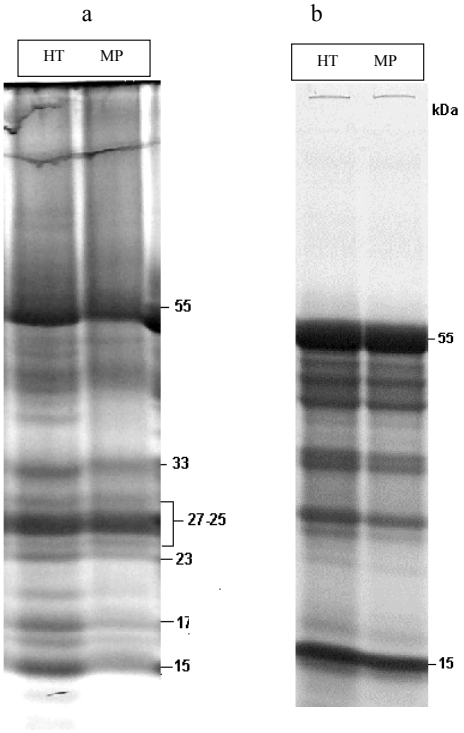

Fig. 5: Chl fluorescence analysis of leaves

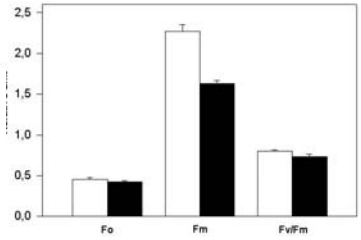

Fig. 6: analysis of various electron transport chain

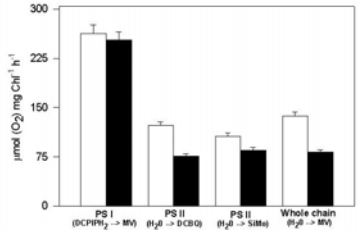

Fig. 7: analysis of various electron transport chain

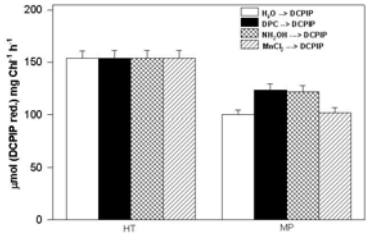

Table Mean values ± std. dev. of some morphological and chemical variable of leaves (blade and petiole).

Characteristics	MP	HT
leaf's weight (g)	5,12 ± 0,09	4,79 ± 0,08
blade's weight (g)	4,23 ± 0,19	3,88 ± 0,09
petiol's weight (g)	0,88 ± 0,09	0,91 ± 0,01
vein's length (mm)	135 ± 4,2	135 ± 0,7
petiol's length (mm)	98 ± 8,4	103 ± 4,2
SPAD index	33,9 ± 0,1 **b**	37,6 ± 0,0 **a**
N blade (% s.s.)	2,24 ± 0,33	2,56 ± 0,11
P blade (% s.s.)	0,28 ± 0,03	0,26 ± 0,06
K blade (% s.s.)	1,65 ± 0,01	1,59 ± 0,06
Ca blade (% s.s.)	2,49 ± 0,32	2,92 ± 0,38
Mg blade (% s.s.)	0,32 ± 0,01	0,32 ± 0,03
Fe blade (% s.s.)	76 ± 7	88 ± 20
Mn blade (% s.s.)	174,5 ± 21,9	194,5 ± 57,3
Bo blade (% s.s.)	26 ± 1,4	27 ± 2,8
Zn blade (% s.s.)	34,5 ± 3,5	39 ± 15,5
Cu blade (% s.s.)	410 ± 209	410 ± 249
S blade (% s.s.)	0,16 ± 0,0	0,20 ± 0,01
N petiol (% s.s.)	0,49 ± 0,03	0,51 ± 0,03
P blade (% s.s.)	0,63 ± 0,12	0,51 ± 0,07
K petiol (% s.s.)	4,78 ± 0,25	4,70 ± 0,08
Ca petiol (% s.s.)	1,92 ± 0,40	2,00 ± 0,44
Mg petiol (% s.s.)	0,62 ± 0,14	0,61 ± 0,15
Fe petiol (% s.s.)	12,5 ± 0,7	13,5 ± 2,1
Mn petiol (% s.s.)	82 ± 7	95 ± 5

Note: different letters of the same variable given significantly differ (p 0.05) by Tukey's test

SANITATION OF WINE GRAPE SELECTION FROM CENTRAL AND SOUTHERN ITALY

G. Bottalico, A. Campanale, P. La Notte, C. Pirolo and V. Savino

Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy

Sanitation of grapevines from virus diseases is currently done either by conventional methods [heat therapy (HT) and meristem tip culture (MTP), separately or in combination] or by more sophisticated techniques (somatic embryogenesis, micrografting, and cryotherapy) (1, 2, 3, 4, 5). Knowing the behaviour of the etiological agents of the disease can be essential for the choice of the sanitation technique to be adopted. We now report. the results obtained in a three-year sanitation trial by MTP or HT of wine grape accessions selected in Apulia and Marche (cental and south-eastern Italy).

One hundred and twenty eight candidate clones belonging to 55 grape wine cultivars were selected for the sanitation trials. All accessions were previously assayed by ELISA for the following viruses: *Grapevine fanleaf virus* (GFLV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV) and *Grapevine leafroll-associated virus 1*, *3*, and *7* (GLRaV-1, GLRaV-3, and GLRaV-7). The infection rate was very high, up to 95-100% for GVA and the closterovirids . According the virus species and based on previous experience (2), HT and/or MTP was carried out on *in vivo* plants or *in vitro*-grown explants. Meristem tips of 0.4 -0.6 mm in size were excised from young 4-5 cm-long shoots of greenhouse-grown vines and plated on the establisment medium (6). HT *in vivo* was on potted grapevines at 38 °C for 60 to 120 days. After this treatment, meristem tips were explanted. Variable periods of *in vitro* heat therapy at 38 °C on 2 cm-long shoots that had been grown *in vitro* for 3 months were also tried for 20, 40 and 60 days. Starting from the 3rd month after *in vivo* acclimatation , ELISA tests were done 3 times a year.

Sanitation rate by MTP for the Apulian cultivars averaged 94.6%, with percentages varying from 91.4% for GVA, 92.4 % for GFKV, 77.7 % for GFLV and 99,5% for the closterovirids, whereas the overall sanitation rate of selections from the Marche was 76.9%.

Sanitation rates combining HT *in vivo* and meristem tip explants reached 93.6% for vines kept at 38° for 60 days, 96.3% for 90 days and 93.9% for 120 days. The elimination of GFLV from 96.1% of the accessions treated for 90 days, is noteworthy. Plants heat reared *in vitro* at 38°C for 20 days, gave a sanitation rate of 96.5%.

The results of this investigations show the efficiency of MTP when applied in large scale sanitation programs. In particular, MTP is by itself able to eliminate vitiviruses and closterovirids to a high percentage. By contrast, heat therapy treatment seems to be necessary for knocking out GFLV, regardless of whether infected vines plants were grown *in vivo* or *in vitro*.

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