AMERICAN VINEYARD FOUNDATION

FINAL REPORT - JUNE,1993

Project Title:

Meristem tip culturing for the elimination of grapevine viruses.

Principal Investigator: Deborah A. Golino

EXECUTIVE SUMMARY:

The new grapevine importation and quarantine facility at FPMS has begun accepting orders this winter. When the next step is the construction is complete, grapevine virus elimination services can be added to the program. The most important technical problem which limits the importation of winegrape clones and rootstocks is the difficulty with which grapevine viruses are eliminated using the old heat therapy techniques. These techniques are slow and inefficient. A number of laboratories worldwide have reported that meristem tip culturing is an effective technique for eliminating grapevine viruses. We have developed those those techniques in our laboratories and are evaluating their effectiveness. This approach should result in streamlining of procedures for the elimination of grapevine viruses from valuable grape propagating materials. As a result of these streamlined procedures, the length of time required for entry of infected materials should be substantially reduced. In turn, this should provide greater accessibility to foreign materials which include European wine clones, improved rootstocks which are needed for many reasons including their potential phylloxera resistance and other products of worldwide grapevine breeding programs. Furthermore, these techniques should further the efficient therapy for virus elimination of important California field selections suspected or proven to be infected with grapevine latent viruses.

With the increased funding available during the 1992-1993 research year it was possible to hire a technician trained in tissue culture research on other crops for this project. This has allowed us to recapture the momentum which was lost in the 1991-1992 cycle when only part-time student help was available for the work. Many of the objectives of this project have been accomplished. In the cases where the work is complete or nearly complete, I have attached the scientific papers which document the research. Some of these manuscripts are not completed in which case the prospects for finishing the projects is discussed in the corresponding appendix.

The technique of shoot tip culture seems to be sucsessful. Treated explants will be monitored to insure that virus does not reappear. Additional future work will focus on improving survival rates and efficiency.

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Summary of Results:

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The Davis grapevine virus collection has been established (Appendix 1). This collection is important to the meristem tip culture research as well as future grape virology research at Davis.

The research by my graduate student Raquel Salati on the use of ELISA testing of tissue culture plants is complete and a full research article has been submitted this month for publication (Appendix 2). This work established that reliable ELISA testing of tissue culture plants is possible, opening up the possibility of early screening of microtip plants which have been through virus elimination therapy. The use of this type of screening should greatly increase the efficiency of *in vitro* elimination programs.

Also, the experiments on the use of the chemotherapeutic Ribavirin are nearly complete. The tissue culture part of the work, which is the most difficult and time consuming, is finished and we are waiting for the results of final ELISA tests when the young plants are 6 months old so that we can publish our results (Appendix 3).

The experiment to eliminate virus(es) from the four Napa valley Cabernets have been successful according to ELISA testing conducted last fall. An abstract of this work has been submitted for the upcoming ASEV meetings (Appendix 4). The original virus-infected vines from these vineyards have been propagated in the Davis Grapevine Virus Collection; the newly produced healthy grapes should be large enough to be planted next fall. The part of the work which is related to this project is complete; we are very interested in following up with clonal evaluation of the healthy

and diseased materials at the Oakville field station when sufficient wood is available.

The experiments to determine the optimal explant size for virus elimination remain to be completed. Plants have been regenerated for explants from < .3 mm through larger microtips and nodes for all 10 virus infected selections used for this experiment. Survival for the smaller explant sizes is still disappointing. We have begun cutting meristem tips with the goal of increasing the numbers of explants produced for each size class of each virus infected grape selection so that the data resulting from this work is statisticly significant and establishes parameters for practical applications of this work. Many of the microtip propagated plants are negative for virus when ELISA tested so we are encouraged about the application of this work to virus elimination programs.

Objectives and timetable:

1. Obtain necessary biological materials for experiments i.e. construct collection of certified grape varieties with and without fanleaf, leafroll and corky bark. (Completed).
1988-1989

2. Develop tissue culture techniques for production of grapes from meristem tip cultures. (Completed).
1988-1989

3. Culture from virus-infected materials. Use molecular assays to do preliminary determination of viral status of explants; set up bioassays in indicator varieties. (In progress).
1989-1993

4. Read bioassays of meristem tip propagated materials.
1993-1995

5. Set up field plots to determine the effects of meristem tip propagation upon cultivar qualities.
1996

Research Accomplishments:

GRAPEVINE VIRUS COLLECTION:

This work is nearing completion and is described in the AJEV technical brief entitled "The Davis Grapevine Virus Collection" (Appendix 1)

Strains of all the major grapevine viruses of quarantine significance have been located using the old Goheen-FPMS records. Dormant wood from those virus infected vines was been collected and chip budded to the virus-free plants which will constitute the new Davis grapevine virus collection.

Bench-grafted vines of Thompson seedless Davis selection 02A, Cabernet Sauvignon Davis selection 05, and Chardonnay Davis selection 04 have been propagated and were chip budded with the virus strains mentioned above. All are grafted onto the FPMS Saint George selection 15 which has been chosen for its freedom from viruses (it is self-indexing for many of the most important ones) and the vigor it will provide, producing plentiful wood for grapevine virus research.

These grapevines have been planted in a field site on the UC Davis campus and are growing well. A list of the individual vines and their virus status is included as appendix 1. Healthy indicator vines for the traditional field index are in Row 1 as a guard row: Cabernet Franc, St. George, LN 33-1 and Baco Blanc. The collection now includes 20 different strains of viruses inoculated into three vines in all three cultivars, with room to add four more virus strains.

In addition, own rooted vines of the three varieties listed above and St. George alone have been chip budded and are awaiting planting in the collection this summer.

The source plants of each of the 21 virus strains has been chip budded to indicator varieties in Davis by both Golino's lab and FPMS. Additional indexing will be done in Canada and New York to insure the identity of all the virus strains.

The plants were originally chip budded the Spring of 1989 while they were in containers with two chip grafts, one from a bud and one from an internode. After the vineyard was planted data was taken throughout the summer on the effectiveness of the inoculation. This was done by a visual check on bud growth. In the fall, the buds were disbudded and the chips were read to see if they had healed in. If the cambium was still green, it was assumed that the virus had been successfully transferred from the chip to the healthy vine. The vineyard was pruned back to two bud spurs during the dormant season. Replacement vines were planted as needed in Spring 1990. If none of the previous grafts healed properly, the vine was reinoculated in Spring of 1990 by field side-grafting. An additional strain of leafroll was added to the collection since a new antisera to this particular strain was developed in the last year.

This vineyard is already providing us with genetically identical material differing only in its viral status. We also work with other available grape collections for genetically identical material. Davis has three large grape collections; one in the Viticulture Dept., Foundation Plant Material Services, and the National Clonal Germplasm Repository. The virus indexing records from Dr Goheen's program has data on many of the plants in these three collections. Currently, we have identified 32 pairs of genetically identical grapes differing by only their viral status. The original vine was diseased and the virus-free vine successfully went through heat eradication. Dormant wood was collected from

many of these vines. When there are large established vines of these pairs the wood has been used for tissue culture experiments and has been re-indexed to verify the viral status. In some cases the vines were very weak and in temporary planting sites. These plants have been propagated and will be planted later this summer. These materials will not replace the new virus collection which is in viticulturally useful varieties, unlike almost all of the "accidental pairs" we have discovered.

Dormant wood was also collected from all of the original virus source plants. This wood was used to re-inoculate the plants that required it and also to index all of the source vines.

An additional collection of own rooted vines of diverse sources of the grapevine viruses has been propagated. Twenty-nine pairs of genetically identical grapes which differ only in viral status have been identified surviving in the Davis grape collections at FPMS, the USDA-ARS clonal repository and in the Viticulture and Enology Department vineyards. These pairs came into being when individual vines were cured of disease by heat treatment during the Goheen program and, largely due to circumstances, the original virus infected source plant survived in one or another collection.

A system for identifying the grapevine virus isolates has been established and accepted by a number of my colleagues. This will allow for better international cooperation and communication as research developments are made using this type collection. As molecular techniques develop for detection and elimination of these viruses, their practical utility can be judged against this type collection.

DEVELOP TECHNIQUES FOR MERISTEM CULTURE OF GRAPES:

Culturing from explants: Experiments have been conducted on the ability of meristems to survive and grow in our laboratories. As reported previously, routine culture of explants <0.5 has been possible. Since this is the range of size reported to eliminate many of the grapevine virus and virus-like disease, we are proceeding to determine the largest possible explant which can be cultured and still reliably eliminate each of the grapevine viruses.

With this goal, a large experiment has been conducted with virus isolates/grape selection combinations which will be subjected to culturing from explants of the following sizes: <0.5 mm, 2-3 mm, and 1 node cuttings (See appendix 5 for varieties, numbers and size of explants). This will allow us to determine the size of explant which can used to eliminate each virus, since each is unique and would be expected to differ in terms of tissue distribution, titer, etc. Appendix 1 is a list of those selections of virus infected materials which are being used for these experiments. Large collections of dormant wood, with appropriate combinations of grapevine viruses, were stored this winter and are being used in the year's experiments. In addition, we have found that early

spring growth can be successfully propagated directly from the field.

This is highly encouraging information. Although it is possible that the viruses are still present but at very low concentrations, and re-testing will need to be done over the years, this data appears to demonstrate that the meristem tip culture is working for virus elimination. The full data sets are not yet complete and ELISA testing is not possible for some viruses like Rupestris Stem pitting virus, but so far it seems that the techniques are working.

CHEMOTHERAPY

The effectiveness of using the viral inhibitor, ribavirin, to eliminate grapevine viruses **in vivo** and **in vitro**: Ribavirin has been reported to inhibit the replication of some plant viruses. For some plants, this has meant that combining Ribavirin treatments with tissue culture virus elimination techniques has greatly increased the success rate of the procedure. For this experiment we are using Cabernet Sauvignon for the Davis Grapevine Virus Collection. We found the several different varieties we used last year produced inconsistant growth; the availability of materials from the collection has greatly facilitated the progress made this year.

Experiments were conducted by where the same virus infected cultivars were excised as nodes and put directly upon media containing Rivavirin at varying concentration. This approach has been used very successfully in the tissue culture production of virus free potatoes. The strategy is to grow the explants for a relatively short period of time on ribavirin containing media and then subculture a presumably virus free tip onto normal media. The same concentrations (0, 50, 200, and 800 ppm) that were used for foliar applications were used for our first tests of this approach. The higher concentrations proved highly toxic and tests are now underway in tissue culture at 0. 10, 50, 100 and 200 ppm. This work is nearly complete; the time consuming tissue culture part of the work is done. (See appendix 3 for details.)

FIELD TESTING: We feel that field testing of the experimental material generated by this program should be conducted. This testing would involve field plots identical to those used for clonal evaluation. We have chosen as materials virus infected selections from several well respected Cabernet Sauvignon vineyards for our experiments.

Over the years, certain vineyards have developed a reputation for quality based on the fact that excellent wines have been made from the grapes which are harvested from those vineyards. Scion wood from vineyards with a history of providing the grapes which go into fine wines is often used for propagating new vineyards based on this wine making history.

Many factors are known to contribute to the eventual performance of

a vineyard. The clonal identity of the scion wood is one factor. In addition, the disease status of selection may have an effect upon yields and quality.

With the permission of the owners, we have harvested dormant wood from the Tokalon vineyard of Mondavi, Martha's vineyard of Tom May, the See vineyard at Silverado and the old Inglenook vineyard on the Coppola property. In each vineyard, four vines have been marked and pruning wood collected. Each has been indexed this winter using the woody indicator system used by FPMS. In addition, ELISA testing of the materials has shown that three of these selections are infected with strains of leafroll virus which can be detected with the available leafroll antisera.

These selections are now completed tissue culture therapy. They have been tested individually with ELISA and will be further tested by teh woody indexing techniques. The clones will be available as FPMS clean selections. More important, each selection will be retested with it's virus-infected parent material to determine the combined effects of virus infection and meristem tip culture.

Field plots would be planted according to the most successful plot designs used to evaluate clonal variability in cooperation with Dr. Wolpert. It is our intention to take these experiments through wine quality trials.

These tests should prove the utility of the tissue culture virus elimination techniques which we are testing and provide data on the effects of virus diseases upon wine quality. In addition, we intend to make separate evaluation of the effects of the techniques alone upon variability of explants which are virus free before being subjected to tissue culture techniques. Reports from other groups suggest that a minimum of change is introduced into meristem cultured plants; prudence would dictate that this potential problem be addressed early in any program attempting to introduce the technique into routine virus elimination procedures. (See Appendix 4)

DEVELOP MOLECULAR ASSAYS FOR GRAPEVINE VIRUSES:

Working in collaboration with Dr. Adib Rowhani, the principle investigator on an AVF grant to develop antisera to grapevine viruses for FPMS, good progress has already been made in optimizing ELISAs, a quick sensitive serological test, for a number of the grapevine viruses (Grapevine fanleaf virus, tomato ringspot virus, and some strains of leafroll). Through that grant and this one, it will be possible to use ELISAs in combination with meristem culturing in the upcoming year, providing quick feedback on the success of our virus elimination work as it is in progress.

A major breakthrough has been made this year for grapevine virus detection. A new technology called polymerase chain reaction (PCR) has been developed which allow extremely sensitive detection of nucleic acid including plant viruses. This technique is much more sensitive than ELISA. USDA purchased a PCR machine for my lab but

efforts to use standard PCR protocols for grape tissue did not work; we believe this is because substance(s) in the grape tissue inhibited the PCR enzymes. My colleague, Adib Rowhani, deserves a great deal of credit for his contribution to this project. Dr. Rowhani developed a new buffer and extraction protocol which is successful. As a result, we can now detect fanleaf virus at extremely low levels. (We need DNA clones and/or sequences of a virus before we can use PCR. These are only available for fanleaf at this time but the cloning project mentioned above may provide them for other grape viruses soon.) This has opened up new areas of research which should be very helpful both to the grape clean stock program and to the rootstock breeding program.

Four sample extraction methods for infected plant tissues were compared. GFLV was readily detected in samples of GFLV RNA and GFLV-infected leaf samples of Gomphrena globosa using all four extraction methods, however only method 4 (detailed in enclosed manuscript) was useful for GFLV detection in grape tissue. Extracts from healthy tissue prepared by methods 1, 2, and 3 prevented detection of extracted GFLV genomic RNAs by PCR m demonstrating that grape tissue extracts can inhibit PCR reactions. Using method 4, GFLV could be detected in all tested cultivars of European grape, Vitis vinifera , and an American species, Vitis rupestris. Detection was possible in infected leaves, shoots, roots and bark scrapings. Dilution of infected grape leaf samples by a 200 fold excess of healthy leaf tissue was not sufficient to prevent GFLV detection. PCR detection of GFLV specific bands was possible for samples containing as little as 64 pg/ml of purified GFLV-RNA (See appendix 5).

My graduate student, Marissa Maningas, is working of cloning using dsRNA as a template. She has produced clones in the winter of 1993 and is now in the process of determining the specificity of those clones. If specific for corky bark virus, they should greatly speed up and increase the sensitivity of testing.

ADDITIONAL FUNDING:

The USDA-ARS has purchased 4 growth chambers (@ $6,000 each) as a high priority request for this research. These have been received, installed, and are in use at this time. With this additional growth chamber space, it has been possible to expand the scale of this project.

$10,000 each has been granted by the California Raisin Advisory Board and $5,00 from the California Table Grape Commission for a related project on rapid detection techniques for grapevine virus indexing.

A sucsessful grant proposal was written to BARD, the Binational Agricultural Research and Development Fund, in an effort to obtain additional funding for this project. We have received sufficient funding to pay a graduate student, Marissa Maningas, in the upcoming year who will be working on techniques for the

sensitive detection of corky bark virus.

Outside Presentations of Research:

The results of these experiments will be published as articles in scientific and popular journals. I frequently lecture for university extension classes and at workshops for farm advisors and growers. In addition, I intend to be available to FPMS, CDFA and others with an interest in grapevine clean stock programs for the training of their personnel in these techniques. I hold the APHIS departmental permit for the importation of grapevines at Davis and, therefore, serve as administrative director of the National Grapevine Importation Program at Davis, through FPMS. This facilitates the implementation of research results in practical programs as developed.

Some publications relevant to this project and available upon request:

Assam, O.I., Gonsalves, D. and Golino, D.A. Detection of DsRNA from grapevines infected with Rupestris stem potting disease and the variabilities encountered. (In Press.)

Bosica, D., Hu, J.S., Golino, D., and Gonsalves, D. 1989. Characterization of grape leafroll associated closterovirus (GLRaV) Serotype II and comparison with GLRaV Serotype III. Phytopathology 80:117. (Abstract)

Bosica, D., Hu, J.S., Golino, D., and Gonsalves, D. 1990. Characterization of grapevine leafroll associated closterovirus (GLRaV) Type II and comparison with GLRaV type III. (In preparation for Plant Disease)

Bovey, R., Caudwell, A., Frison, E.A., Golino, D.A., Gonsalves, Ikin, R., Kyriakopoulou, T., Martelli, G.P., Rezaian, M.A., Rumbos, I., Walter, B. FAO/IBPGR Technical Guidelines for the Safe Movement of Grapevine Germplasm. Eds. E.A. Frison and R. Ikin. In Press.

Golino, D., Uyemoto, J. and Goheen, D. 1992. Grape Virus Diseases. In "Grape Pest Management". Agricultural Sciences Publications, University of California, Berkeley.

Golino, D.A., Foster, J., Gonsalves, D., Rosenburg, D. and Tolin, S. 1988. A report on the current status of grapevine importation and quarantine. Written by the WRCC-24 committee grapevine importation and quarantine, accepted by the WRCC-24 and submitted to the grape CAC. 28 pp.

Golino, D.A. 1988. Grapevine Importation in California: At a crossroads. California Plant Pathology No. 82.

Golino, D.A. 1989. Grapevine Importation: the Process and the Problems. Practical Winery and Vineyards X (1):81-84.

Golino, D.A., Rowhani, A. and Walker, A. Grapevine Fanleaf Virus in the Vineyard. For Practical Winery and Vineyard. (In Press).

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Namba, S., Bosicia, D. Azzam, O., Maixner, M. Hu, J.S., Golino, D., and Gonsalves, D. Purification and properties of closterovirus-like particles isolated from a corky bark diseased grapevine. Proceedings of the 10th meeting of the international council for the study of viruses and virus disease of grapevine, Volos Greece. In Press.

Rowhani, A. 1992. Use of $F(ab')_2$ Antibody Fragment in ELISA for detection of grapevine viruses. Am. J. Enol. Vitic. 43:38-40.

Rowhani, A., Golino, D., and Cunningham, M. 1992. Comparison of bioassay indexing and ELISA for the detection of grapevine leafroll virus in grapevine selections. Phytopathology 82: 1148. (Abstract).

Salati, R. 1992. Monitoring grapevine closteroviruses associated with leafroll, corkybark, and Rupestris stem pitting using serological and dsRNA techniques. M.S. Thesis. Department of Plant Pathology, University of California, Davis, Ca.

Salati, R., Golino, D.A., Rowhani, A. and Gonsalves, D. 1992. ELISA detection *in vitro* of grapevine latent viruses associated with leafroll and corky bark diseases. American Journal of Enology and Viticulture 43: 309.