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Isolation, Serological Detection of Closteroviruses Associated with Grape Corky Bark, and ELISA-dsRNA Analysis to Monitor Grape Leafroll Elimination Procedures

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Isolation, Serological Detection of Closteroviruses Associated with Grape Corky
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TABLE OF CONTENTS

ABSTRACT.....	2
OBJECTIVES OF GRANT.....	3
RESULTS AND DISCUSSION.....	3
Characterization of closteroviruses associated with grapevine corky bark.....	3
Production and use of antibodies to closteroviruses associated with grapevine corky bark.....	7
Effectiveness of ELISA for monitoring virus elimination methods; and the rapid <i>in vitro</i> diagnosis of corky bark.....	9
Cloning of dsRNA associated with leafroll infected vines and characterization of cDNA clones for detection and genome analysis.....	12
DESCRIPTION OF COOPERATION.....	14
EVALUATION OF RESEARCH ACHIEVEMENTS.....	14
PUBLICATIONS.....	17
MANUSCRIPTS IN PRESS, SUBMITTED, OR IN PREPARATION.....	19
APPENDED PUBLICATIONS AND MANUSCRIPTS.....	21

ABSTRACT

The objectives of the grant were to: 1) purify and characterize closteroviruses associated with grapevine corky bark (CB), 2) produce monoclonal and polyclonal antibodies to closteroviruses associated with grapevine CB, 3) determine the effectiveness of ELISA and dsRNA techniques for monitoring virus elimination methods, using grapevine leafroll associated viruses for the model system, and 4) initiate development of nucleic acid probes that are associated with grapevine leafroll. All of the objectives were accomplished and extended. A virus collection was established to preserve and serve as virus sources for a number of grape viruses. Long (1400-2000nm) and a shorter (800nm) closterovirus-like particles were isolated from a CB infected 'Semillon'. Antisera were produced to the long (CB100) and short particles (GVB). Most of the subsequent work was then concentrated on CB100. Antiserum to CB100 were used to monitor and establish virus elimination procedures for *in vitro* cultured grapevines. Serological studies showed that antiserum to CB100 reacted with some CB infected vines in California and Israel but not with others. Although the etiology of CB still remains unclear, our work has prompted research activity on CB in a number of other laboratories. A rapid diagnostic method for CB was developed by *in vitro* micrografting. Insect transmission tests showed that CB100 was transmitted by mealybugs. DsRNA from leafroll diseased vines was cloned and nucleic acid probes developed for leafroll detection. About 60% of the 20kb genome of leafroll was sequenced and comparative analysis with other closteroviruses showed that the genome is organized similarly to beet yellows virus. These research results will help control corky bark by establishing serological detection methods and a rapid biological indexing method, developing *in vitro* virus elimination methods, and allowing for the comparisons of CB isolates in different geographic regions. The establishment of a new grape virus collection at Davis will give access of standard virus isolates to investigators. Information on the genome of leafroll will help with the characterization of this important virus and eventually allow the development of transgenic plants that may be resistant to leafroll virus.

OBJECTIVES OF GRANT

- 1) To purify and characterize closteroviruses associated with grapevine corky bark.
- 2) Produce monoclonal and polyclonal antibodies to closteroviruses associated with grapevine corky bark.
- 3) To determine the effectiveness of ELISA and dsRNA techniques for monitoring virus elimination methods, using grapevine leafroll associated viruses for the model system.
- 4) Initiate development of nucleic acid probes that are associated with grapevine leafroll.

RESULTS AND DISCUSSION

Characterization of closteroviruses associated with grapevine corky bark

This part of the grant was carried out in the three laboratories. Gonsalves' laboratory was responsible for purifying and characterizing of closteroviruses associated with corky bark; Golino's laboratory supplied materials for the purification, established a virus collection that would serve to preserve various virus isolates, and worked on cloning of dsRNA of corky bark (CB). Tanne's laboratory worked on the serological association of CB in Israel with the CB virus characterized in the U.S., worked on cloning of dsRNA of CB, and on the use of dsRNA as a diagnostic tool for CB.

Establishment of Grapevine Virus Collection at Davis. This very important collection was started by Golino to establish a permanent source of virus material that would be available to investigators throughout the world. This collection is especially valuable because many of the grape viruses are defined simply by their reactions on indicator plants. The following section on the virus collection was written by Golino.

Much of this work is based on the materials which are being developed in the new Davis Grapevine virus collection. A manuscript (Golino, D. 1992. The Davis Grapevine Virus Collection. American Journal of Enology and Viticulture. 43: 200-205.) which describes this collection in detail has been published and is included with this report.

Briefly, the collection is an organized group of grapevine virus isolates. The isolates have been located using the historic Davis indexing records. Many of the virus isolates are endangered because they are being systematically eliminated from other grape collections and replaced with virus free vines; in addition, the single small grapevine virus

collection which has existed on campus is scheduled for removal. Continuing research progress on the control of the grapevine viruses requires that standard virus type isolates remain available for characterization and comparison. We feel that this is a pivotal time in creating this collection, since much of this viral genetic material will not be available shortly. To insure the success of this grant, a special emphasis was been placed on locating and preserving vines which have indexed positive for CB and leafroll disease. All isolates in this collection have been assigned unique isolate numbers which are being used to distinguish between accessions.

Some isolates have been preserved as vines produced by cuttings on their own roots. These vines were propagated last winter and planted this spring in the virus source vines collection. A few important selections, including the leafroll isolates and CB isolates which react with the antisera produced in Gonsalves' laboratory, have been chip budded into healthy vine of commercial varieties in what is being called the clonal virus collection. This collection should provide Cabernet Sauvignon, Thompson Seedless, and Chardonnay vines with several of these important leafroll and CB type isolates which can be used by both Golino and Tanne in the virus elimination work and provide Gonsalves with valuable raw materials for the development of both serological and DNA probes.

Purification of viruses associated with corky bark disease. This work was carried out in Gonsalves' laboratory. The initial work concentrated on purifying and characterizing closteroviruses isolated from the standard CB isolate from Davis which is designated CB100 in Golino's virus collection. The CB100 infected 'Semillon' has served as a positive control for the indexing program at Davis for many years. We successfully isolated long closterovirus-like particles 1400-2000nm in length. Further work showed that this closterovirus like particle had a coat protein molecular weight of 24,000 daltons. This work is detailed in the attached publication by Namba et al. 1991. Purification and properties of closterovirus-like particles associated with grapevine corky bark disease. *Phytopathology* 81: 964-970. This was the first publication on the isolation and characterization of a virus from CB diseased vines. Antiserum (details given below in another section) was produced to the virus particles and used extensively by Tanne and Golino in this grant. The isolated virus was designated CB100.

The work on virus purification described above also led to other very interesting and important results. We were able to mechanically transmit a virus from CB100 infected 'Semillon' to the herbaceous plant *Nicotiana occidentalis*. The virus particles associated with infection on *N. occidentalis* were 800nm long and did not react to antiserum prepared

to CB100. This virus was further characterized in a publication (attached to report) by Boscia et al. 1993. Properties of a filamentous virus isolated from grapevines affected by corky bark. Arch. Virology 130: 109-120. Antisera was produced to it and the virus was subsequently named GVB. There is evidence that GVB is also associated with CB. Work is being carried out on this virus by Boscia and colleagues in Italy.

The above works sparked intense interests and speculations on the cause of CB because two types of viruses were isolated from CB100 vines and different tests suggested that the CB disease may be caused by more than one virus. The etiology of CB is still not resolved but the work from this BARD grant was a key towards focusing efforts by a number of laboratories to determine the cause of CB.

Characterization of corky bark associated closterovirus infections in Israel. This work was carried out in Tanne's laboratory. A 1400nm closterovirus was purified from corky bark (CB)-infected grapevines in Geneva, and antiserum (CB100) was made against it. This antiserum was used to characterize and monitor CB infections in Israel. Direct and indirect ELISA tests were performed with CB100 antiserum in CB-indexed and field samples exhibiting CB symptoms. Introduced material which was positively indexed as CB-infected was also included in these tests. An Israeli type isolate of CB reacted positively with antiserum to CB100. However, only some of the field material which had been indexed as CB-infected reacted with CB100 antiserum. Moreover, samples from plants which had been diagnosed as leafroll-infected, occasionally reacted with CB100. It is concluded that the "phenotype" of the CB disease is determined not from a single type of virus infection. However, the CB100 virus is definitely also associated with CB in Israel.

Spread of corky bark disease in vineyards and transmission of corky bark by mealybugs. This work was done and written up by Tanne. Until recently the spread of virus diseases was attributed mostly to transmission through propagative material. We have tested methodically whether the CB disease is being spread in established vineyards. Indicator plants (LN-33, Cabernet Franc, *Rupestris* St. George) were interplanted in a CB-diseased vineyard, and were monitored periodically for CB by symptom appearance and ELISA. Typical disease symptoms have been observed on LN-33 indicators after 6 years, indicating a natural spread of CB. The spread of the disease in the vineyard variety itself (Thompson Seedless) was analyzed statistically. Analysis of the temporal progress and spatial patterns indicated a shift from a clustered foci of infection to a random pattern, supporting a spread within the vineyard from initial foci, probably by a vector.

Transmission of corky bark by mealybugs in Israel. The transmission of CB by mealybugs was first reported by us. At the time of that report we did not have the CB100 antiserum, and infection was determined by symptoms. We have extended the study to include ELISA follow-up of transmission tests. Donor plants were CB-infected grapevine (diagnosed by ELISA with CB100). Non viruliferous mealybug crawlers of the 1st and 2nd stage were given an acquisition feeding of 48 hours, then transmitted to healthy grapevine indicators (LN-33, Rupestris St. George, Cabernet Franc) for inoculation feeding of 4 days. Plants were tested by ELISA and the virus could be detected in some cases as soon as 6 months after inoculation. Mealybug transmission of CB was thereby confirmed, and a recommendation to apply pesticides in commercial vineyard has been issued and are being carried out. This work was done in collaboration with Dr. Y. Ben-Dov and Dr. B. Raccach.

A proposed comparative study of CB isolates from Israel and California was not carried out, since the Californian plant material was confiscated and destroyed by the Israeli Plant Protection Authorities.

Double-stranded RNA as a diagnostic tool for leafroll and corky bark infections. This work was done and written up by Tanne in Israel. Since our previous work showed that double-stranded RNA (dsRNA) was detected in diseased tissues at a low incidence, we modified the method by standardizing the source tissue (dormant bark scrapes), performing the extraction in an uninterrupted manner (not pausing after every step), and adding activated charcoal to the extraction solution. These modifications greatly improved the rate of detection, and a good correlation has been established between indexing and dsRNA analysis. Although, this is a laborious and time-consuming method, it can still be effectively used when a small-scale, reliable diagnosis is required, such as the follow-up of a mother plant stock.

Efforts to clone the dsRNA from corky bark infected grapevines. Although cloning of dsRNA from CB infected grapevines was not an original objective of the grant proposal, efforts were made in this area since it was felt that cDNA clones could provide a more definitive diagnosis of the disease. This is especially true with grape virus diseases since many of them, including CB, are defined by reactions on grape indicator plants. These efforts were carried out in the laboratories of Tanne and Golino. Below are summaries of the efforts by both laboratories.

Golino. The efforts to clone dsRNA from CB100 was carried out by an M.S. graduate student, Marissa Maningas, at the University of California at Davis. The work is given in her thesis entitled "The development of molecular-based detection techniques for strawberry mild yellow edge and grapevine CB disease". An attached manuscript entitled "Efforts to clone corky bark associated virus isolate 100 from dsRNAs purified from infected vines" has been prepared for submission to American Journal of Enology and Viticulture. In brief, none of the cloning efforts to produce CB cDNA were successful. In an effort to get 'purer' forms of CB, the mealybug *Pseudococcus longispinus* was used to transmit CB from infected 'Semillon' to the indicator LN-33 and two herbaceous host plants, tomato and *Vinca rosea*. Transmission of CB was successful to LN-33 but not to tomato and *Vinca*.

Tanne. It is very difficult to get sufficient amount of purified grapevine closteroviruses from which good quality RNA can be isolated to start a cloning procedure. Therefore we have started cloning from dsRNA obtained by the modified procedure described above. The dsRNA preparation, after DNase and RNase treatments, was warmed to 80 C in 50% formamide. A mixture of random hexanucleotides was added, and the reaction mixture was allowed to cool down slowly to 37 C. The annealed structures were then subjected to reverse transcription followed by polymerase chain reaction (PCR), and cloned into the plasmid Bluescript. Selected clones are now in the process of verification and analysis.

Another virus, GVA, was also found in CB-diseased plants. Therefore, we initiated its cloning as well. Specific primers were obtained from Dr. Minafra (Bari, Italy) and a 433-base long cDNA clone was made from dsRNA and sequenced. The relevance of the clone to the causative agent of the disease was established by probing infected grapes and *Nicotiana benthamiana* with this clone. The isolated clone contains an Open Reading Frame, and the translated peptide was checked against the GenEmble data base. The GVA peptide sequences did not correspond with any sequence on file.

Production and use of antibodies to closteroviruses associated with grapevine corky bark

Production of antiserum was done in Gonsalves' laboratory and was reported in the publications by Namba et al. 1991. Phytopathology 81: 964-970 for the long (1400-2000nm) closterovirus-like particles and by Boscia et al. 1993. Arch. Virology 130: 109-

120 for the shorter (800nm) closterovirus like particles. Antiserum to the longer virus particles (CB100) were used extensively throughout this grant. The antiserum to the shorter particles was used less since it was produced at the latter stages of the grant and since Boscia (following his stay at Geneva) took on the project after returning to Italy.

The antiserum to CB100 was used in Israel to characterize CB infections under field condition and to determine the optimal time for sampling tissues for detection of CB by ELISA. At Davis, the antiserum was used to monitor tissue cultured plants (this aspect is presented in another section) for the effectiveness of chemotherapeutic agents for eliminating viruses from *in vitro* culture of grapevines. It was used in Gonsalves' laboratory to characterize the CB100 particles by western blotting and by electron microscopy (Namba et al. 1991. Phytopathology 81: 964-970). Below are specific uses of the antiserum by Tanne's and Golino's laboratories.

Tanne. Various factors that affect the diagnosis of grapevines for CB100 were examined. These are as follows:

- (a) The type of the tested tissue for best detection. Leaf petioles, leaf blades, bark scrapes and roots were analyzed by ELISA. CB was detected in all tissues, but the best tissue that also correlated best with indexing was bark scrapes from dormant plants. The results with roots is somewhat ambiguous, and it is not recommended to use them for detection assays.
- (b) A possible seasonal and other time factors effect on detection. Age of infection was found to be a factor in detection. Indicator plants were infected with a defined CB source either by grafting or by mealybugs. ELISA tests with CB100 were performed periodically during 3 years. At the first year CB could be detected only at the 9th-12th leaf stage, while during the second year infection could be detected in some plants already at the 4th-5th leaf stage, and in the third year most of the plants could be positively diagnosed at the 4th-5th leaf stage. This build-up of infection is probably due to virus accumulation over time (in contrary to many other infections where fluctuation and even recovery is common). In CB infections, old infections are easier to detect then new ones.
- (c) Diagnosis in tissue culture. Explants from infected Thompson Seedless vines were established in cultures as calli or as shoots. ELISA tests were performed periodically on these tissues. CB was hardly detected in calli cultures, but was readily detected in shoot cultures even after 3-4 subculturings.

Golino. Antiserum to CB100 was used extensively in monitoring the distribution of the virus *in vitro* by ELISA, and in monitoring virus elimination steps by ELISA. These

results are given in another section. Furthermore, the antiserum is now used for routine ELISA testing of grapevines in the Foundations Plant Material Services collection at Davis.

Effectiveness of ELISA for monitoring virus elimination methods; the rapid *in vitro* diagnosis of corky bark

Work under this section was done in the laboratories of Tanne and Golino. Monitoring the virus elimination methods was done for leafroll and also for CB.

Golino. Progress has been made on several projects which address this objective.

In vitro monitoring of grapevine leafroll associated viruses and grapevine corky bark associated virus using an f(ab')₂ ELISA. F(ab')₂ ELISA was used to detect grapevine closterolike viruses in *in vitro* cultures of *Vitis vinifera* cv. Cabernet Sauvignon. One node explants were collected in late summer from vines with leafroll and CB and from uninoculated controls. Sources of infected vines included Leafroll 101, 102, 106, 109, and CB100. Each isolate tested ELISA positive with its homologous antisera. Eight weeks after culture initiation, the cultures were divided into subsamples consisting of petioles and leaves, stems and shoot tips, roots, and original explant nodal tissue. The samples were tested by ELISA. First and second subcultures were propagated and subsequently tested as above. Statistically significant ELISA readings were obtained from all tissue types, but stem and shoot tips produced the highest A405 values. These results indicate that ELISA testing of *in vitro* grapevine node cultures can provide meaningful results in virus elimination programs. This portion of the research is described in the attached manuscript of Salati et al., 1993. "Monitoring of grapevine leafroll associated virus and corky bark associated virus with ELISA *in vitro*" which has been submitted to Plant Disease.

The use of Ribavirin for the elimination of virus from *in vitro* cultures of *Vitis vinifera* cv. Cabernet Sauvignon infected with grapevine fanleaf virus, grapevine leafroll associated viruses, and corky bark associated virus. Three different techniques were used to apply ribavirin to infected grape tissue. The first approach involved foliar application of 0, 10, 50, 200, and 800 ppm solutions twice weekly to dormant cuttings which were being rooted in sand with bottom heat. After 6-8 weeks sufficient growth had developed for 2-4 mm shoot tips to be taken and established as tissue culture explants. The second approach was to take 2-4 mm shoot tips and establish them directly on media which contained ribavirin at 0, 10, 50, 100, and 200 ppm. The third technique involved cutting 1 node

cuttings (1-2 cm) of green growing canes (see below for additional details) and establishing them on media with ribavirin at 0, 5, 10, 20 and 50 ppm; after the nodes had produced 5-8 cm of growth, 2-4 mm shoot tips were taken from these explants and established on standard tissue culture media.

Ribavirin as a chemotherapeutic to augment microtip propagation for the elimination of these viruses must be used in a simple, robust procedure to provide an improvement in current virus elimination technology. Problems were discovered with the first two approaches which were attempted. Foliar application was found to be time consuming and uniform applications were difficult to achieve as the cuttings grew. A long period of time was required before tissue was available to provide explants. The use of media made with varying concentrations of ribavirin was found to simplify the application to infected grape tissue. However, small shoot tips of 2-4 mm developed poorly on media containing even low concentrations of the drug. Therefore, the third approach in which nodes obtained from field grown vines were established on media containing the ribavirin treatment and then subcultured to normal media was chosen for further study. These experiments are discussed in detail in the attached manuscript by Golino et al., 1994. "The Use for the chemotherapeutic ribavirin for the elimination of grapevine viruses from *in vitro* cultures of grapes as detected with F(ab')₂ ELISA." which will be submitted to American Journal of Enology and Viticulture.

Micropropagation for the elimination of grapevine viruses. 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 statistically 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.

Tanne's laboratory was provided with propagating wood in 1991 to begin the research in her lab with the same standard isolates needed for her work. In addition, samples were mailed to her in Beltsville, Maryland, where she had been on sabbatical. Dr. Tanne was able to obtain a permit to import more materials into Israel in 1992 but the Agricultural Commissioner's office in California has changed the rules governing the

issuance of phytosanitary permits and this has made it impossible to ship materials under the old guidelines from the Davis plant pathology farm. Tanne and Golino are in the process of developing new procedures which may allow these shipments to continue.

Tanne. Progress was made in the following areas:

Virus elimination by micropropagation. *In vitro* cultures from CB, leafroll and GVA infected explants were established and served as a source for meristem tip cultures. Meristem tips, 0.2-0.5 mm in size, were excised and grown on paper bridges in 0.5 X MS medium. After 4 weeks the surviving growing meristems were transferred to solid media (still in 0.5 X MS) and developed into shoots. At the stage of 4-5 leaves they were tested by ELISA, and later transferred to Jiffy pots. The meristem-originated plants were tested by ELISA for leafroll (type III), CB and GVA periodically. Presently, 4-6 months after transfer to *in vivo* conditions, none of them have reacted positive to any of the antisera. They seem to be virus-free and will be tested by indexing in the next season.

Rapid diagnosis in vitro. Indexing is the most reliable way for grapevine virus diagnosis, since viral diseases in grapevine seem to be a complex of mixed infections. Hence, the inability to detect a certain virus by a serological or molecular assay may not be indicative of the health status of the plant. Indexing by woody indicators, however, is a laborious and time-consuming procedure causing a "bottle-neck" in the routine of quarantine and new releases of grapevine material. We have developed two methods of *in vitro* indexing, shortening indexing time from years to weeks.

(1) *In vitro micrografting.* Explants from defined infected vines were established as *in vitro* cultures. Healthy indicator-derived cultures were parallelly established. Micrografting of cultured scions derived from infected material on cultured indicators, or vice versa, was performed under aseptic conditions, and the grafted plants were kept in culture. Defined CB symptoms developed on LN-33 8-12 weeks after grafting in 30% of the cases. This work is detailed in the attached publication by Tanne et al., 1993. Rapidly diagnosing grapevine corky-bark by *in vitro* micrografting. HortScience 28: 667-668. Similarly, leafroll symptoms developed in such grafts on Cabernet Franc within 3 months from grafting at a higher incidence than that of CB. Further development to increase the rate of success and shorten indexing time even more is currently underway.

(2) *Enhancement of symptom appearance in vitro.* In the initial *in vitro* studies we noticed that various sugar compositions of the medium had a stress effect on the cultured explant.

This observation helped to initiate a study to provoke early and defined symptoms in explants from diseased plants. This study is in progress.

Cloning of dsRNA associated with leafroll infected vines and characterization of cDNA clones for detection and genome analysis

This work was done in Gonsalves' laboratory. The main objectives of this work were to develop methods for obtaining cDNA clones of grapevine leafroll, to use the clones in various detection methods and to undertake the sequencing of genome of grapevine leafroll. An ultimate practical aim, although beyond the scope of the project, was to develop nucleic acid based diagnostic tests that would detect the range of grapevine leafroll types (I, II, III, and IV).

All work was done with grapevine leafroll type III since Gonsalves' laboratory had previously characterized this isolate. We successfully cloned the dsRNA purified from infected grapevines. A library of cDNA clones was obtained and selected clones sequenced for the development of probes that could be used for detection of leafroll type III. We detected specific nucleic acid from leafroll diseased vines by dot blot hybridization, reverse transcription PCR (RT-PCR), and Immuno-capture RT-PCR. Details of the cloning and detection are in the attached manuscript by Ling et al., 1994, "Molecular cloning and detection of grapevine leafroll virus by nucleic acid hybridization and polymerase chain reaction" that was submitted to Phytopathology.

The library of cDNA clones was mapped by a combination of restriction enzyme digestion analyses and by sequencing the ends of numerous clones and lining up clones with overlapping sequences. We have sequenced about 60% of the genome. Our work has shown that leafroll type III has a genome organization that is similar to other closteroviruses as beet yellows virus (see Fig 1). We believe this is the most extensive sequence analysis of the closteroviruses associated with grapevine leafroll disease.

Efforts are now centered at developing routine and practical ways of diagnosing leafroll, completing the sequence of the genome, and developing nucleic based probes that may detect a range of the leafroll isolates. Eventually, we want to develop transgenic grapevines which express the coat protein gene of leafroll type III.

Figure 1. Partial genome organization of grapevine leafroll associated closterovirus type III (GLRaV III) in comparison to the complete genome sequence of beet yellows virus (BYV), the only closterovirus sequenced entirely thus far. The sequence of GLRaV is positioned appropriately to the BYV genome according to nucleotide homology between these viruses.

DESCRIPTION OF COOPERATION

This project provided a unique opportunity for cooperation between laboratories with different expertise and access to virus infected vines. The collaboration worked very well for the most part. Essentially, Gonsalves' laboratory focused on purification, characterization, and production of antisera to closteroviruses associated with CB virus. This was done primarily because of his previous experience in working with closteroviruses of grapevines (e.g. leafroll). However, this work would not have been possible without Golino's laboratory because it supplied all of the virus source material for purification. Once antiserum was produced, it was used in a variety of works that were done in the laboratories of Golino and Tanne. As noted in this report, they focused on epidemiology of CB, transmission of CB, and monitoring virus elimination methods of CB and leafroll. While Gonsalves' laboratory did not have ready access to virus isolates, Tanne's and Golino's had access to numerous CB isolates and have expertise in virus elimination techniques. Additionally, they were well suited for studying the transmission of CB by vectors and the spread of CB in the fields.

Experiments were planned to have a direct comparison of California and Israel CB isolates by Golino's laboratory sending infected grapevines to Israel. Unfortunately, the materials were destroyed after arriving in Israel because of regulatory restrictions. Efforts are still being made to accomplish this planned exchange of material.

Throughout the project, Gonsalves' laboratory focused on cloning of the dsRNA associated with grapevine leafroll. A similar rationale was used in the collaboration. Once probes were developed and tested in Gonsalves' laboratory, they would be utilized in Golino's and Tanne's laboratories. Again, this was an effective way to collaborate because Golino and Tanne have access to numerous leafroll isolates. Collaboration in these areas will continue beyond this current grant.

EVALUATION OF RESEARCH ACHIEVEMENTS

We completed all of the objectives of the grant proposal and did research that extended beyond the objectives.

The first objective was to purify and characterize closteroviruses associated with grapevine CB. The importance of achieving this objective was in developing an association

of definitive virus particles with the disease. Corky bark is defined as a disease condition that occurs on the LN-33 indicator. This criterion is still the definition but our work now associates it with specific virus particles. Furthermore, the work has sparked wide interest in CB because of the information obtained on the virus particles and because it created possibilities and speculations on which particles are really the causal agent(s) of the CB disease. These developments will help towards the unraveling of the etiology of CB. Much information was obtained on the spread of CB in Israel vineyards, the transmission of CB by mealybugs in Israel and California, and the association of the long particles associated with CB in California and Israel. These works showed that the long particles were associated with some CB isolates but not others. As noted above, these information has sparked much interest in other laboratories towards investigating the etiology of CB.

Work was also done to determine the use of dsRNA for diagnosing CB and to refine the dsRNA isolation procedure further in order to develop routine methods for isolating dsRNA from CB infected grapevines. These methods will be useful for other closteroviruses that infect grapevines.

The second objective involved the development of antisera to the virus particles and was a natural consequence of the first objective. Production of good antiserum with is not a trivial matter because of the very low titer of virus particles and because of presence of healthy contaminants. Once the CB100 antiserum was developed, it led to the effective investigation of CB as outlined in objective three. The development of the F(ab')₂ ELISA technique for CB100 helped to augment the direct and indirect ELISA methods that are used in Tanne's and Gonsalves' laboratory.

Objective three was accomplished and much useful and practical information came out of the research. For one, various ways for eliminating CB and leafroll were established in the Golino's and Tanne's laboratories. Their approaches were different but aimed at the same objective. Their work showed the value of antisera for monitoring and evaluating virus elimination procedures. Additional work also showed that CB could be diagnosed in a relative short time by *in vitro* micrografting. This achievement will certainly help in streamlining the indexing method of CB. Overall, the research on virus elimination and monitoring of the virus elimination steps has provided a good base for development of routine methods of eliminating virus from *in vitro* tissue cultured plants.

Objective four was reached and extended. The development of cloning methods for grapevine leafroll using dsRNA will enable the thorough characterization of grapevine

leafroll. The techniques should also be useful for cloning of CB. We developed nucleic acid based probes for detection grapevine leafroll and have sequenced over half of the 20kb genome of leafroll. Genome analysis showed that the long closterovirus-like particles associated with leafroll type III is indeed a closterovirus. These developments set the stage for use of nucleic acid based diagnostic tests for leafroll and the possibility to develop nucleic acid probes that could be used to diagnose the range of leafroll types.

The practical importance of the research to agriculture are in several areas. Since control of grape viruses is largely accomplished by prevention, great importance is placed on effective diagnosis of grape virus diseases. A series of standard grape indicators are normally used to diagnose grape viruses. Previous research in many laboratories made much improvement on shortening the indexing procedures by using serology and herbaceous indicators. However, three important disease (leafroll, CB, and stem pitting) still require the woody indicators. Much progress has been made in the serological detection of leafroll, but relatively little is known about stem pitting. Our work on CB helped to move the indexing of at least some isolates of CB to the serological stage; it helped define the possible closteroviruses that cause CB; and diagnosis by *in vitro* micropropagation shortened the indexing time to months instead of 2-3 years. The development of nucleic acid probes for detecting leafroll adds another important tool towards the diagnosis of this disease. All of these research accomplishments will aid in the control of CB and grapevine leafroll.

Virus elimination procedures developed in this project will help towards the control of grape viruses making clean material available to growers. Furthermore, importation of grape cultivars constitute a very important way of improving grape industries. Cultivars that are infected need to be cleaned up. The technology developed in this grant helps towards this aspect.

Very little is known about the genome of CB and leafroll. Information on the genome of these viruses would provide basic information to understand and classify these viruses and to develop nucleic based diagnostic tests. Furthermore, recent works have shown that transgenic plants expressing the coat protein gene of a virus are protected against that target virus. Our genome sequencing of leafroll will enable us to locate the coat protein gene and thus provide the possibility of developing transgenic grape plants that resist leafroll. This could be of economic importance in areas where leafroll is rapidly spread by insect vectors.

PUBLICATIONS

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