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**Yellow Disease of Grapevines: Impact, Pathogen
Molecular Detection and Identification, Epidemiology,
and Potential for Control**

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PATHOGEN MOLECULAR DETECTION AND IDENTIFICATION,
EPIDEMIOLOGY, AND POTENTIAL FOR CONTROL**

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ABSTRACT

Grapevine yellows diseases characterized by similar symptoms have been reported in several countries including Israel, the United States, France, Italy, Spain, Germany, and Australia. These diseases are among the most serious known in grapevine, but precise knowledge of the pathogens' identities and modes of their spread is needed to devise effective control strategies. The overall goals of this project were to develop improved molecular diagnostic procedures for detection and identification of the presumed mycoplasmalike organism (MLO) pathogens, now termed phytoplasmas, and to apply these procedures to investigate impact and spread and potential for controlling grapevine yellows diseases. In the course of this research project, increased incidence of grapevine yellows was found in Israel and the United States; the major grapevine yellows phytoplasma in Israel was identified and its 16S rRNA gene characterized; leafhopper vectors of this grapevine yellows phytoplasma in Israel were identified; a second phytoplasma was discovered in diseased grapevines in Israel; the grapevine yellows disease in the U.S. was found to be distinct from that in Israel; grapevine yellows in Virginia, USA, was found to be caused by two different phytoplasmas; both phytoplasmas in Virginia grapevines were molecularly characterized and classified; commercial grapevines in Europe were discovered to host a phytoplasma associated with aster yellows disease in the USA, but this phytoplasma has not been found in grapevine in USA; the Australian grapevine yellows phytoplasma was found to be distinct from the grapevine phytoplasmas in Israel, the United States, and Europe and was described and named "Candidatus phytoplasma australiense"; and weed host plants acting as potential reservoirs of the grapevine phytoplasmas were discovered. These and other findings from the project should aid in the design and development of strategies for managing the grapevine yellows disease problem.

OBJECTIVES

- Objective 1.** Survey yellows disease incidence and severity in the U.S. and Israel, using as criteria yellows disease symptomatology and molecular detection of MLO (now termed phytoplasma) pathogens.
- Objective 2.** Identify and classify group affiliations of individual grapevine yellows associated MLO (phytoplasma) strains using DNA based molecular techniques.
- Objective 3.** Design polymerase chain reactions for specific detection and identification of individual grapevine yellows associated MLO (phytoplasma) strains or groups of strains for detailed analysis of the epidemiology of the disease.
- Objective 4.** Discover and identify potential insect vectors and alternate plant hosts of grapevine yellows associated MLOs (phytoplasmas).
- Objective 5.** Test the efficacy of the antibiotic oxytetracycline for the remission of disease symptoms and the reduction of MLO (phytoplasma) infection in grapevine.

INTRODUCTION

Grapevine is an ancient crop of major economic importance today for the fruit and wine industries. It is grown world-wide in various combinations of rootstocks, varieties/cultivars, and *Vitis* species, in order to adapt to various environmental conditions. The crop is infected by many viruses and "virus-like" diseases, among them the yellows diseases. Symptoms of the grapevine yellows include vein yellowing and necrosis, abortion of flowers and/or fruits, leaf curling, lack of lignification in new shoots, general loss of vigor, and die-back. In some regions, a 20-30%, or greater, crop loss can occur in non-epidemic situations.

The establishment of grapevine certification programs has focused mainly on diseases caused by viruses. Still, "virus-like" diseases continue to affect vineyards and cause heavy damage. Grapevine yellows is reported to be spreading to all viticultural countries and areas, affecting table grapes as well as wine grapes. For yet unknown reasons, the yellows diseases often are expressed in an epidemic form, affecting a great number of grapes simultaneously.

Grapevine yellows appears as one of the most serious problems in grapevine culture and one of the most puzzling diseases. The disease can have devastating effects on crop yield and quality and on vine survival. Although an insect vector of the grapevine yellows disease in continental Europe known as *Flavescence dorée* has been reported, forms of grapevine yellows occur in regions where that insect is unknown, and vectors of grapevine yellows in such latter regions were unknown when this project was begun. Even the naming of grapevine yellows disease(s) has been problematic, with the name *bois noir* being applied in France to a grapevine yellows disease not spread by the vector of the *Flavescence dorée* pathogen.

At the start of this project, available data had indicated that cell wall-less pathogens (mycoplasma-like organisms = MLOs, now called phytoplasmas) belonging to at least three different groups were associated with grapevine yellows disease. Still today, relatively little is known of the geographical limits of these three pathogens, and only since this project was begun has new light been shed on insect vectors of potential importance in the long distance and field spread of phytoplasmas causing grapevine yellows in Israel.

In part as a result of the work accomplished during this project, grapevine yellows is now viewed as a complex of several distinct diseases that are caused by phylogenetically distinct phytoplasmas. This disease complex impacts vineyards on at least three continents. In several countries, including the United States, Israel, Italy, Australia, and France, grapevine yellows is responsible for substantial crop losses and, in many cases, loss of vines. Although phytoplasmas are implicated as the causes of the grapevine yellows diseases, these pathogens cannot be isolated in pure culture *in vitro*, and rigorous proof of their roles in grapevine yellows cannot be achieved.

One of the most critical aspects for understanding and controlling grapevine yellows revolves around the detection and identification of the phytoplasma pathogens, and determination of their mode and extent of spread. In this project, we have used molecular methods for detection, identification, and characterization of phytoplasmas in yellows diseased grapevines and in weeds. The results provide data needed for understanding the impact and epidemiology of grapevine yellows and for targeting alternate host plants and insect vectors to control disease spread.

MATERIALS AND METHODS

Plant samples and reference phytoplasma strains: During 1994, 1995, and 1996, samples of leaves (whole leaves or midribs and/or petioles) were collected from naturally diseased cvs. Chardonnay, Cabernet franc, Cabarne, Carenigan, and Riesling grapevine (*V. vinifera* L.) plants exhibiting symptoms of grapevine yellows in five commercial vineyards located in the Virginia Piedmont area and/or in vineyards in Golan Heights, "Jonathan", Gshur, Kfar Tabor, Kidron, Gedrea, Odem, Yatir, the Arad Valley, and Zor'a in Israel. Samples were also collected from plants of wild grapevine (*V. riparia* Michx.) growing in the vicinity of Virginia vineyards and *Amaranthus*, *Carthamus* sp., and *Convolvulus* sp. in the vicinity of Israel vineyards containing diseased *V. vinifera*, and from healthy seedlings of *V. vinifera* grown in an insect proof greenhouse. For biological assays, and for preservation of tested material, leaves from suspected phytoplasma-infected grapevines were grafted onto periwinkle plants. Reference phytoplasmas used as controls in this study were maintained by graft transmission in plants of periwinkle (*Catharanthus roseus* [L.] G.Don) maintained in a greenhouse.

Monitoring of vineyards for possible insect vectors of grapevine yellows. Yellow sticky traps were used to collect insects in and around vineyards that were affected by grapevine yellows in Israel. Several traps were placed in each vineyard and surrounding area that was studied. Each trap was exposed in the field for a period of one week, and new traps were placed in the field at two-week intervals. Insects that had adhered to the surfaces of the traps were gently removed and were identified according to species. The data that were obtained were analyzed to determine species of insects, principally leafhoppers, that were present in and around vineyards, and to determine the changes in insect population throughout growing season.

The traps were placed in a field in the following manner. A network of sticky traps was situated at ten trapping stations. Each station contained six traps placed in a straight line and a group of trap plants (30-50 plants of each of the following: potato, safflower, broadbean). The traps were arranged as follows: one trap meter outward from the trap plants, one trap in the middle of the trap plants, and the remaining four in the vineyard proper, six meters distant from each other. The first two traps were about 10 cm above the canopy of the trap plant (about 50 cm from the ground) and those in the vineyard were 10 cm above the grapevine canopy. Later in the summer additional traps were posted on the same poles, but leveled at 10 cm above the ground for local, rather than migrating, leafhoppers. Leafhoppers were collected from the trap plants using a portable suction trap.

Primer pairs and conditions for polymerase chain reaction (PCR):

Nucleic acid for use as template in PCR was extracted from fresh tissue by previously described methods (Maixner et al. 1995; Prince et al. 1993). After extraction, DNA was purified using GeneCleanIII kit as specified by the manufacturer (Bio101, Vista, CA).

Seven pairs of oligonucleotide primers were used in PCRs. R16mF2/R16R1 and R16F2n/R16R2 are phytoplasma universal primer pairs (Gundersen and Lee, 1996). Primer pairs R16(I)F1/R16(I)R1, R16(III)F2/R16(III)R1, and R16(V)F1/R16(V)R1 prime specific amplification of 16S rDNA from phytoplasmas belonging to groups (*sensu* Lee et al. 1993) 16SrI, 16SrIII, and 16SrV, respectively (Lee et al. 1994a). Primer pair rpF1/R1 primes amplification of phytoplasma ribosomal protein (rp) gene operon sequences (Gundersen et al. 1994; Lim and Sears, 1992). Primer pair rp(III)F1/rp(III)R1 primes amplification of rp gene operon sequences from group 16SrIII phytoplasma strains (Davis et al. 1998).

Both a single (direct, nonnested) PCR and nested PCR were used. In one nested PCR protocol, DNA amplified in PCR primed by R16mF2/R16R1 was diluted 1:50 with sterile distilled water and used as template in PCR primed by R16F2n/R16R2. In a separate nested PCR protocol, DNA amplified in PCR primed by R16F2n/R16R2 was diluted 1:50 with sterile distilled water and used as template in PCR primed by R16(I)F1/R16(I)R1, R16(III)F2/R16(III)R1, or R16(V)F1/R16(V)R1. Primer pairs rpF1/R1 and rp(III)F1/rp(III)R1 were used in direct, non-nested PCR only. All PCR was carried out as previously described (Jomantiene et al. 1998).

16S rDNA from Canada X-disease, elm yellows, and aster yellows phytoplasmas was amplified in PCR from templates extracted from diseased plants of periwinkle (*Catharanthus roseus*), and the cloned DNAs were used as positive internal controls.

Restriction fragment length polymorphism (RFLP) analyses of PCR-amplified DNA: Products from nested PCR primed by R16F2n/R16R2 were analyzed by single enzyme digestion, according to manufacturers' instructions, with Aci I, BamHI, BfaI, DdeI, DraI, EcoR2, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, MseI, MspI, RsaI, Sau3AI, TaqI, and ThaI (New England Biolabs, Beverly, Mass.) and AluI (Life Technologies Inc., Gaithersburg, MD). The digested DNAs were analyzed by electrophoresis of the digestion products in a 5% polyacrylamide gel, followed by staining with ethidium bromide and visualization of DNA bands with a UV transilluminator. The RFLP patterns of Virginia grapevine yellows phytoplasma DNAs and of the Israel grapevine yellows phytoplasmas were compared with the RFLP patterns obtained using reference phytoplasmas in this study and the RFLP patterns previously published (Davis et al. 1997a,b; Lee et al. 1993; Lee et al. 1998; Prince et al. 1993; Vibio et al. 1996). Phytoplasmas detected in this work were assigned to 16S rRNA gene groups and subgroups as previously described by Lee et al. (1993, 1998).

Nucleotide sequencing, sequence alignment, and putative restriction site analysis: PCR-amplified 16S rRNA gene and rp gene operon products were sequenced using standard procedures. The nucleotide sequence determined for phytoplasma VGYIII rDNA was deposited in the GenBank database (GenBank, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland). Other sequences used in this study were obtained from GenBank. A partial sequence of clover yellow edge (CYE) phytoplasma 16S rDNA (Accession no. L33766) was obtained from GenBank. In the present work, we amplified and sequenced 16S rDNA from clover yellow edge phytoplasma in order to verify the GenBank 16S rDNA sequence from this clover yellow edge phytoplasma and to complete nucleotide sequencing of 42 bases missing from GenBank Accession no. L33766. 16S rRNA gene sequences from Virginia grapevine yellows phytoplasma VGYIII and from other group 16SrIII phytoplasmas were analyzed to identify recognition sequences for selected restriction enzymes. Putative restriction site maps were generated by using the DNASTAR program MapDraw option (DNASTAR Inc., Madison, Wisc.). Alignments of sequences were generated and sequence similarities evaluated by using the DNASTAR program Megalign option.

RESULTS

Objective 1. Survey yellows disease incidence and severity, using as criteria yellows disease symptomatology and molecular detection of MLO (now termed phytoplasma) pathogens.

Four vineyards were surveyed annually in order to record the appearance of yellows symptoms on grapevines, with the aim of analyzing the disease spread in the vineyards in Israel. Three plots were selected in the north, center and south of the Golan Heights, and one plot was surveyed in the south of the country. Doublets Analysis, Ordinary runs and Quadratic-Variance methods were applied to investigate the spatial dispersion patterns of grapevine yellows. Doublets Analysis and Ordinary Runs were applied to the experimental plots in the south and north of Golan. Quadratic-Variance was used to analyze the data from a different plot in the north of the Golan. The disease symptoms spread to nearly all grapevines in the central plot and the plot was therefore abandoned. Partial analysis (using the same statistical methods) was performed in the vineyard growing in the south of the country. The results of spatial analysis (Fig. 1) suggest that the grapevine yellows disease is transmitted from vine to vine in an aggregated manner. Clustering is more obvious in cultivars that are sensitive to the disease (e.g., Chardonnay), whereas in less sensitive cultivars the clustering is less pronounced. The spatial analysis and clustering of infected grapevines tend to suggest that the disease spread is due to vector infection.

Samples from symptomatic and asymptomatic plants were collected from different growing regions and from various cultivars (most of them wine varieties) in Golan. Midribs and petioles were used to extract DNA according to Maixner et al. (1995). PCR analysis was conducted using primers specific for a 16S rDNA region conserved in all phytoplasmas. These universal primers were synthesized according to Lee et al. (1993), and nested PCR primers used were synthesized according to Lee et al. (1994). In some cases, primers fU5/rU3 according to Lorenz have been applied. Table 1 is a summary of the results obtained by PCR analysis of wine and table grape varieties in various regions using universal primers.

PCR analysis demonstrated the presence of phytoplasma in many wine cultivars in all grapevine growing regions in Israel. In 3 to 4 years, the disease spread all over the country. We could not detect phytoplasma in plants that did not exhibit symptoms. In one case phytoplasma was detected in a table-grape variety growing close to a Chardonnay vineyard.

In order to enhance the study of grapevine phytoplasmas, graft transmission to periwinkle was attempted. Shoot tips of infected grapevines were grafted onto healthy periwinkle plants; DNA from these plants was extracted and analyzed by PCR. Disease-transmission experiments were carried out by grafting scions from yellows-exhibiting grapevines onto periwinkle. Scions were taken from yellows-exhibiting grapevines collected from various parts of Israel. In 43% (36/85) of the cases, the grafted periwinkle developed disease symptoms within 3 months. Grafting scions from noninfected grapevines did not produce symptoms in periwinkle. All symptomatic donor grapevines were checked by PCR with universal primers to determine whether they carry phytoplasma. The same test was performed with periwinkle DNA. All of the symptom-developing grafted periwinkle plants were found to carry phytoplasma. Typing experiments were carried out to verify that the transmitted phytoplasma faithfully represented the form of the pathogen existing in the donor grapevine. In general, two kinds of typing were performed: group typing by nested PCR using group-specific primers, and typing by restriction analysis of the various group-specific PCR products. The phytoplasmas transmitted from grapevine to periwinkle were of the same groups and

subgroups as those found in the donor grapevines. Restriction analysis of the various nested PCR products with a variety of enzymes indicated that the amplified phytoplasma DNAs from grapevine plants and the respective periwinkle plants were very similar or identical to one another (Tanne and Orenstein, 1998).

In the Piedmont region of Virginia, USA, yellows diseased grapevine plants in five vineyards were investigated. During each of three growing seasons, leaves and young shoots were excised from symptomatic grapevine plants and analyzed in the laboratory for the presence of phytoplasma. More than 250 samples were analyzed during the project. Preliminary studies had suggested that two different phytoplasmas were present in the plants, and that one phytoplasma might be more prevalent during the early part of the season, whereas, the second phytoplasma might predominate late in the growing season (J.P. Prince and R.E. Davis, unpublished). The results from further, extensive analyses clearly showed that this hypothesis was not correct. Both phytoplasmas were present throughout the season, but one phytoplasma (group 16SrI, subgroup A) was found in only one vineyard; the group 16SrIII subgroup I phytoplasma was found only in the four other vineyards. Both phytoplasmas were detected in plants about one week before the onset of visible symptoms and remained detectable in diseased plants throughout the season. The increase in disease incidence observed during the three year study appeared to be most obvious in the four vineyards where the group 16SrIII subgroup I phytoplasma was discovered.

Objective 2. Identify and classify group affiliations of individual grapevine yellows associated MLO (phytoplasma) strains using DNA based molecular techniques.

PCR was used as the means to amplify 16S rRNA gene sequences for analysis of phytoplasmas present in yellows diseased grapevines surveyed in Israel and the United States. Phytoplasmas infecting grapevines with yellows diseases in other regions of the world were included for comparisons, because identification and characterization of possibly different pathogens in grapevines elsewhere is critical for devising rational approaches for the production of disease-free planting materials and for establishing effective grapevine yellows disease quarantine measures.

Identification and classification of the Israel grapevine yellows phytoplasma was based on the PCR analysis of DNA extracted from symptomatic grapevines (Davis et al. 1997). In further investigation of the Israel grapevine yellows disease, work was carried out to determine the frequency of plant infection and to determine whether or not any phytoplasma in addition to the stolbur (group 16SrXII-A) phytoplasma may be present in diseased grapevines in the region. This further investigation was prompted by the finding of mixed phytoplasma infection in grapevines elsewhere (Alma et al. 1996). Universal primers were applied to amplify 16S rDNA at the first stage of analysis (Fig. 2). 16S rRNA group affiliation was confirmed by using group-specific primers for nested PCR (Fig. 3). RFLP analysis of the amplified DNA fragments revealed that a great number of the detected phytoplasmas are members of the aster yellows MLO genomic cluster (*sensu* Lee et al. 1993), which contains the stolbur phytoplasma. Surprisingly, some of the DNA fragments analyzed by RFLP were members of the X-disease group (Figs. 4 & 5). These DNA fragments have been cloned and are now being sequenced in order to finalize the classification of this second phytoplasma found in diseased grapevines in Israel.

Detection and classification of phytoplasmas in diseased cultivated grapevines in Virginia revealed grapevine infections by two different phytoplasmas. Phytoplasmas belonging to two groups, 16SrI and 16SrIII, were detected in grapevine yellows-diseased

V. vinifera in Virginia on the basis of phytoplasma-specific DNA amplification in PCR (Davis et al. 1998). These results confirmed earlier findings by Prince et al. (1993).

Preliminary RFLP analysis of amplified 16S rDNA indicated that the group 16SrI phytoplasma was similar to both subgroup I-A and subgroup I-B phytoplasmas; further analysis by digestion of amplified DNA using Hha I revealed that this phytoplasma was most closely related to subgroup A (type strain, tomato big bud [BB] phytoplasma). RFLP analysis using 12 different restriction enzymes did not distinguish the group 16SrI phytoplasma, designated VGYI, from BB phytoplasma (Davis et al. 1998). There was no evidence indicating the presence of a stolbur-related phytoplasma in the grapevines std in Virginia.

Classification of the group 16SrIII phytoplasma strains in diseased, cultivated grapevines was accomplished using 19 restriction enzymes for RFLP analysis of PCR-amplified 16S rDNA. All strains yielded the same RFLP patterns (Davis et al. 1998). Of the 8 subgroups previously described in group 16SrIII (Lee et al. 1998), the group 16SrIII Virginia grapevine yellows phytoplasma was most closely related to Canada peach X-disease (subgroup III-A), walnut witches' broom (subgroup III-G), spirea stunt (subgroup III-E), and poinsettia branch-inducing (subgroup III-H) phytoplasmas. The VGYIII phytoplasma could be distinguished from poinsettia branch-inducing phytoplasma by digestion of PCR products with Hpa I or with Dde I. VGYIII phytoplasma could be distinguished from Canada peach X-disease phytoplasma by digestion of PCR products with Mse I, from walnut witches'-broom phytoplasma by Rsa I and from spirea stunt phytoplasma by Dde I and Hha I (Davis et al. 1998). RFLP patterns of walnut witches'-broom and spirea stunt phytoplasma DNAs were unusual in the presence of "extra" DNA bands; that is, the total of fragment sizes was considerably larger than that expected for the approx. 1.2 kbp PCR product. We explain these results as composite patterns of 16S rDNA amplified from two rRNA operons, that exhibit interoperon sequence heterogeneity, in both walnut witches'-broom and spirea stunt phytoplasmas. We did not obtain RFLP evidence of two rRNA operons of different sequence in VGYIII, Canada peach X-disease, or poinsettia branch-inducing phytoplasmas. Since the collective RFLP patterns of 16S rDNA from Virginia grapevine yellows phytoplasma VGYIII were unique, we assigned this phytoplasma to a new 16S rRNA subgroup, designated I, in group 16SrIII.

Nucleotide sequence and putative restriction sites in a 16S rRNA gene sequence from Virginia grapevine yellows phytoplasma VGYIII were determined. The nucleotide sequence determined for the 16S rDNA amplified from VGYIII phytoplasma in PCR primed by F2n/R2 was deposited in the GenBank database as Accession number AF060875. The nucleotide sequence of 16S rDNA from VGYIII phytoplasma was compared with the corresponding sequences from western X-disease phytoplasma (Accession no. L04682), clover yellow edge phytoplasma (Accession no. L33766), and Vaccinium witches'-broom phytoplasma (Accession no. X76430). Comparative analyses of putative restriction sites in the sequenced DNA was carried out. Expected fragment sizes based on analysis of putative restriction sites were in excellent agreement with fragment sizes obtained by enzymic RFLP analysis of amplified 16S rDNA. Sequences from Western X-disease and clover yellow edge phytoplasmas, representatives of subgroups 16SrIII-A and 16SrIII-B, respectively, and of Vaccinium witches'-broom phytoplasma, a member of subgroup 16SrIII-B, were distinguished from Virginia grapevine yellows phytoplasma VGYIII by the restriction site analysis (Davis et al. 1998).

Since a grapevine yellows disease identical in symptomatology to the grapevine yellows diseases in Israel and the United States had been observed in eastern Europe, we also investigated whether phytoplasma may be the cause of this disease. The results revealed that grapevines suffering from yellows disease in some regions of eastern Europe are

infected by the same phytoplasma (stolbur phytoplasma) that predominates in diseased grapevines in Israel. This phytoplasma has been known to cause diseases in vegetable crops including tomato and pepper, and its wide geographical distribution suggests it could be of increasing concern in several important crops including grapevine. Thus, we now know that what is learned about the grapevine yellows disease in eastern Europe may aid in combatting the stolbur phytoplasma-induced grapevine yellows in other regions, including Israel. (Kolber et al. 1997)

A preliminary study of grapevine yellows phytoplasmas in northern Italy revealed that grapevine yellows is a complex of at least two diseases that are caused by, respectively, phytoplasmas related to the stolbur phytoplasma and the Flavesence dorée (group 16SrV, elm yellows phytoplasma group) phytoplasma. It was evident from these results that the epidemiology of grapevine yellows in this region differs substantially from that of grapevine yellows in either Israel and the United States, because of the apparent absence of the Flavesence dorée phytoplasma in these latter regions. At the same time, the results pointed out that both phytoplasmas can occur in the same vineyard, and that it continues to be necessary to monitor vineyards for possible invasion by the Flavesence dorée phytoplasma. (Bianco et al. 1996)

During the course of this study, a phytoplasma was detected in diseased plants of strawberry in Florida, and its 16S-23S rRNA spacer region sequence was found to be very similar to that of stolbur phytoplasma (N.A. Harrison, personal communication), raising the possibility that a stolbur phytoplasma might be present in the United States. Because of the importance of stolbur phytoplasma in inducing diseases of numerous plants, including grapevines, in Europe and Israel, we incorporated the strawberry phytoplasma into our investigations. The results of our study indicated that this strawberry phytoplasma was not a member of the stolbur phytoplasma subgroup and that it was instead a member of the Mexican periwinkle virescence phytoplasma group (Jomantiene et al. 1998). We obtained no evidence for the presence of stolbur phytoplasma strains in the United States.

Objective 3. Design polymerase chain reactions for specific detection and identification of individual grapevine yellows associated MLO (phytoplasma) strains or groups of strains for detailed analysis of the epidemiology of the disease.

Ribosomal protein gene operon primers were designed. In order to confirm the presence of group 16SrIII phytoplasmas in some grapevine plants in Virginia, an oligonucleotide pair was designed to prime PCR-mediated amplification of ribosomal protein gene operon sequences from group 16SrIII phytoplasmas. The primers have the following designations and sequences: rp(III)F1, 5'-CTATTGCAAATCAGATGTCTG-3' and rp(III)R1, 5'-AATAAACGAGTTAAACGAAGACC-3'. Conditions of PCR using these primers were the same as those described previously (Jomantiene et al. 1998.). A group 16SrIII-specific DNA fragment of 244 bp was amplified in direct (non-nested) PCR from template DNAs derived from all group 16SrIII phytoplasmas in this study, including the VGYIII Virginia grapevine yellows phytoplasma DNA in extracts of naturally diseased, field-grown grapevine plants. No group III-specific DNA amplification was observed when template DNAs were derived from non-group III phytoplasmas. RFLP analysis of amplified rp gene operon sequences using *AluI* and *MseI* did not distinguish VGYIII from those of Canada peach X-disease, walnut witches'-broom, or spirea stunt phytoplasmas. The amplified 244 bp fragments of the ribosomal protein gene operons from VGYIII and SP1 phytoplasmas were sequenced, and the sequences were aligned with those from group 16SrIII phytoplasmas associated with Canada peach X-disease, western X-disease, and clover yellow edge (Davis et al. 1998). The VGYIII rp gene operon sequence was identical to that of Canada peach X-disease phytoplasma and was differentiated from those of spirea

stunt, western X-disease, and clover yellow edge phytoplasmas by base substitutions in from one to three positions.

Since a group 16SrIII phytoplasma was discovered in diseased grapevines in Israel during the closing stages of this project, primer pair rp(III)F1 and rp(III)R1 will be used in continued work to monitor the geographical distribution of the group 16SrIII phytoplasma in diseased grapevines in Israel.

Objective 4. Discover and identify potential insect vectors and alternate plant hosts of grapevine yellows associated MLOs (phytoplasmas).

Trapping of suspected leafhopper vectors of the grapevine yellows in Israel was begun in April. Leafhoppers collected were *Neoliturus fenestratus*, *Hyalesthes obsoletus*, *Circulifer* spp., and 55 other species. The three named predominated. Peak populations were collected of *Neoliturus* and *Hyalesthes* during June. Leafhopper species collected from trap plants in the field predominantly were: *Circulifer* spp. and *Hyalesthes* on potato; fewer of these species were collected on safflower and broadbeans.

A. Alternate plant hosts.

Numerous species of wild plants were investigated as possible sources/reservoirs of the phytoplasma strains causing grapevine yellows disease symptoms in commercial grapevines. Investigation of wild grapevine (*V. riparia*) plants growing close to infected commercial vineyards in Virginia indicated that wild grapevine, as well as commercial *Vitis* wine cultivars, were infected by a group 16SrI phytoplasma. RFLP analysis distinguished the phytoplasma strains in wild grapevine as members of subgroup I-A, the same subgroup discovered in one of the five commercial wine vineyards studied in Virginia. In addition, plants of *Prunus serotina* growing within 20 meters of diseased vineyards were found to be infected by a group 16SrI phytoplasma that was not distinguished from the phytoplasma found in one of the five vineyards studied. However, no group 16SrI phytoplasma was discovered in those vineyards in proximity to the infected *P. serotina*.

Other results that were obtained indicated that plants of *Carthamus* sp., *Amaranthus* sp., and *Convolvulus* sp., growing in and around vineyards, are infected by phytoplasmas and may act as sources of phytoplasmas for infection of vineyards in Israel.

B. Inoculation time in the vineyard.

For this purpose, we followed two directions:

1. Trapping and monitoring of potential vectors in and around the vineyard.
2. Dispersion of bait plants in infected vineyards.

1. In the final year of the project, yellow sticky traps were placed at three different vineyard locations in Israel: (a) a 17 year infected Cabernet Sauvignon vineyard; (b) a 7 year old Merlot and Cabernet Sauvignon vineyard with little infection; (c) a 4 year old Cabernet Sauvignon vineyard (no detectable infection). Weeds such as *Carthamus* sp. and *Amaranthus* sp. were growing in and around the vineyards. The traps were placed in the vineyards each time for a period of one week, and replaced every two weeks from mid July until September.

The following plant leafhoppers were collected and monitored for seasonal fluctuations: 1-15 July 1997, a few *Hyalesthes obsoletus* and few *Neoliturus guttulatus*. 16-31 July 1997, 22 *H. obsoletus* and over 200 *N. guttulatus*.

The number of leafhoppers declined dramatically in September; no N. guttulatus and only a few H. obsoletus were collected.

2. Healthy periwinkle (Catharanthus roseus) plants were placed in border rows of an infected vineyard located in the center of the country (cultivars Carignan, Chenin blanc, French colombard, and Cabernet Sauvignon). The plants were cycled in the vineyard for a period of one month, between June to October, and brought back to a screen house in Bet-Dagan, where they were monitored for symptoms and were analyzed by PCR to detect possible infection by phytoplasmas.

Periwinkle plants developed disease symptoms such as yellowing of the leaves, small leaves, profuse axillary growth - "witches' broom, and vitrification of the flowers, within 4-6 months after exposure in the vineyard. DNA extractions from leaf tissue of these plants (with and without symptoms) was performed according to the method described by Maixner et al. (1995), and PCR analysis was carried out using universal primers and group-specific primers (Lee et al. 1993,1994). Phytoplasma was detected in plants exhibiting symptoms and not in symptomless plants. Aster yellows type (group 16SrI, sensu Lee et al. 1993) and western-x type (group 16SrIII) phytoplasmas were detected using nested-PCR on this DNA. In parallel, DNA was extracted from grapevines exhibiting yellows symptoms growing in the vineyard. Types of phytoplasma detected by PCR analysis of grapevines corresponded to the ones detected in the periwinkle. The majority of infected plants became inoculated in the field during July and September.

The tentative conclusions of this experiment are:

- (i) Phytoplasmas associated with grapevine yellows detected in bait plants-periwinkle and plant/leafhoppers trapped in these vineyards are probably identical to the ones detected in grapevines (Figure 6).
- (ii) Phytoplasmas associated with yellows disease was spread mainly by vectors that were within the vineyard.
- (iii) H. obsoletus and N. guttulatus are possible vectors of these phytoplasmas.
- (iv) In 1997, the highest population of insects was trapped in July, and a second smaller "wave" (of H. obsoletus) was in September.

In order to understand the epidemiology of this disease, weeds growing in and around infected vineyards have been analyzed by PCR. Phytoplasmas were detected in Carthamus sp., Amaranthus sp., and Convolvulus sp. These weeds may be alternate hosts for the phytoplasmas that cause grapevine yellows.

C. Monitoring insect population in and around vineyards.

A network of sticky traps was placed in two locations in the Golan Heights and one location in the center of the country. The traps were placed for one week and replaced every second week. Leafhoppers were collected, identified, and PCR-analyzed to detect phytoplasmas (Table 2).

The highest population of H. obsoletus in the Golan in 1997 was in July and a second small "wave" at the end of September/October. A large population of N. guttulatus was trapped in June 1997. These two insect species are possibly vectors of grapevine yellows in Israel. Based on the data collected and analyzed in these experiments, there may be two inoculation perids, at least in the Golan Heights.

Objective 5. Test the efficacy of the antibiotic oxytetracycline for the remission of disease symptoms and the reduction of MLO (phytoplasma) infection in grapevine. (This objective was modified because of legal restrictions related to antibiotic usage. Thus, study of the effect of mechanical barriers to reduce disease spread was initiated.)

This Objective was modified to test the efficiency of agrotechnical methods to reduce disease spread. A newly planted (2 years old) Chardonnay vineyard, growing in the north of the Golan Heights, was monitored for development of disease symptoms. The plants were sampled randomly and analyzed by PCR. No symptoms were recorded, and PCR analysis was negative for all tested plants. Periwinkle plants were planted on the borders of the vineyard, but most of them were lost. One single plant tested positive for phytoplasma in PCR. This experiment is planned as a long term trial and will continue for the next 3-4 years to determine invasion of the vineyard by the grapevine yellows pathogen and to evaluate potential means to stem the spread of the disease.

GENERAL DISCUSSION OF THE RESEARCH PROGRAM

Progress was made on all Objectives, including for Objective no. 5, which was modified because of legal restrictions related to antibiotic usage. This progress brings us closer to development of safe and effective strategies for management of the grapevine yellows disease complex. In addition, the progress has yielded new fundamental understanding of the relationships among the various grapevine yellows phytoplasmas and has led to recognition and characterization of previously unknown phytoplasmas.

The use of molecular criteria, particularly the analysis of conserved gene sequences, has permitted the development of a comprehensive phytoplasma classification system (Gundersen et al. 1994; Lee et al. 1993,1998; Seemiller et al. 1994) and an emerging phytoplasma taxonomy. The recognition of 16S rRNA gene groups and some subgroups as distinct species is giving rise to the designation of "Candidatus Phytoplasma species". Thus far, two have been named, "Candidatus Phytoplasma aurantifolia" (Zreik et al. 1995) and "Candidatus Phytoplasma australiense" (Davis et al. 1997a). Most phytoplasmas have not yet been named as "Candidatus species", and group and subgroup designations continue to be highly useful in the current molecular systematics. In particular, subgroup classification of the phytoplasma strains infecting Vitis species may have significance for understanding the epidemiology of grapevine yellows. This project achieved the detection, identification, and classification of phytoplasmas associated with grapevine yellows diseases in Israel, the United States, and other regions.

This work revealed the presence of subgroup XII-A (stolbur phytoplasma subgroup) phytoplasma strains and a group 16SrIII phytoplasma of unknown subgroup in yellows diseased commercial grapevines in Israel, and it revealed subgroup 16SrI-A and 16SrIII-I phytoplasmas infecting diseased grapevine plants in Virginia. Subgroup XII-A phytoplasma strains are also found in yellows diseased grapevines growing in other Mediterranean countries. Subgroup 16SrI-A has been previously described (Lee et al. 1993), and subgroup 16SrIII-I (III-I) is a newly described subgroup. Neither of these latter two subgroups had been previously determined to infect Vitis species.

Our classification of the apparently predominant (stolbur) phytoplasma in diseased grapevines Israel provides clear and extensive documentation of the presence of this phytoplasma in this region of the world. Other results, determined in parallel with these investigations, indicate the presence of this phytoplasma also in diseased potatoes in Israel (E. Tanne and M. Klein, unpublished).

Our classification of the group 16SrI Virginia grapevine yellows phytoplasma in subgroup I-A expands the known plant host range of members of this subgroup and sheds new light on grapevine yellows in North America. It also distinguished this phytoplasma from the stolbur phytoplasma, which previously was also classified in group 16SrI (Lee et

al. 1993). While some phytoplasma strains infecting cultivated grapevine (*V. vinifera*) and wild grapevine (*Vitis* sp.) plants in Virginia were previously identified as members of group 16SrI, their subgroup affiliation was not reported (Prince et al. 1993,1994). Subgroup 16SrI-A (tomato big bud [BB] and related phytoplasmas) is known only in North America and is distinct from subgroup 16SrI-B (aster yellows and related phytoplasmas) (Lee et al. 1993). While subgroup 16SrI-B is known in North America, it has not been reported in *Vitis* spp. in North America, although it has been reported in *V. vinifera* in Europe (Alma et al. 1996). The present communication reports the first work to document that both cultivated and wild grapevine plants are infected by subgroup 16SrI-A strains. Distinction of the group 16SrI Virginia grapevine yellows phytoplasma at 16S rRNA subgroup level distinguishes this phytoplasma from others known to infect *Vitis* species and provides new data that should aid the search for insect vectors and alternate plant hosts that play a role in its spread in Virginia. Subgroup 16SrI-A has a wide plant host range, including wild species native to Virginia and the surrounding region. Subgroup 16SrI-A (I-A) phytoplasmal infections of wild grapevine, gray dogwood (*Cornus racemosa* Lam.), fleabane (*Erigeron canadensis* L.), goldenrod (*Solidago* spp.), and other plants (Davis et al. 1998; Griffiths et al. 1994; Lee et al. 1993,1994a,b) point to several possible sources of subgroup I-A phytoplasma strains that may be transmitted to cultivated grapevines.

While group 16SrIII phytoplasma strains have been reported in *V. vinifera* and *V. riparia* in Virginia (Prince et al. 1993,1994), subgroup affiliation of such strains had not previously been reported. Although other natural plant hosts of group 16SrIII phytoplasmas are known in the region of eastern North America including Virginia (Lee et al. 1993; Gundersen et al. 1996), no plant host other than *V. vinifera* (Davis et al. 1998) has been shown to harbor phytoplasma strains belonging to subgroup III-I.

Other work has also indicated *V. riparia* as an alternate plant host of phytoplasmas that may infect cultivated *V. vinifera*. Chen et al. (1993) detected a phytoplasma in *V. riparia* growing near yellows-diseased cultivated grapevines in New York. On the basis of serological reactions and dot blot hybridizations, they found that this phytoplasma was related to an unidentified grapevine yellows phytoplasma from northern Italy. Based on available data, it is not known whether the phytoplasma reported by Chen et al. (1993) in *V. riparia* in New York is related to either of the phytoplasmas in *Vitis* species in Virginia (Davis et al. 1998). Maixner et al. (1993) reported that *S. titanus* collected from *V. riparia* and *V. vinifera* in New York contained an antigen that was serologically related to flavescence dorée phytoplasma on the basis of results from enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy using polyclonal antiserum. Plants of *Vicia faba* L., but not plants of *V. vinifera*, that had been fed upon by such insects developed symptoms typical of infection by a phytoplasma. Although Maixner et al. (1993) did not identify the phytoplasma antigen presumably acquired by *S. titanus* from *V. riparia* and *V. vinifera*, their work also points to wild *V. riparia* as a possible source of phytoplasma(s) that may infect cultivated grapevine.

In addressing the question of possible relationships between grapevine yellows in North America and grapevine yellows diseases in other parts of the world, it is important to note that neither of the grapevine-infecting phytoplasmas in Virginia has been reported outside of North America. Conversely, no phytoplasma associated with flavescence dorée disease or bois noir disease in Europe, or with Australian grapevine yellows disease, has been reported in North America. Although *S. titanus* in New York was reported to carry a phytoplasma serologically related to the flavescence dorée phytoplasma in France, the published data (Maixner et al. 1993) do not make it possible to identify the phytoplasma or to classify it in any 16S rRNA group.

While the available data including the results from this project do not refute the hypothesis of a North American origin of flavescence dorée phytoplasma as originally proposed by Caudwell (1983), there is no evidence that this phytoplasma, which is a member of group 16SrV (elm yellows and related phytoplasmas, *sensu* Lee et al. 1993) (Prince et al. 1993), occurs anywhere in North America. To date, subgroup 16SrV-A (V-A) is the only subgroup of group 16SrV thus far reported in North America, although differences in ribosomal and nonribosomal DNA have been noted among group V strains (Griffiths et al., 1993). By contrast, subgroups V-A (elm yellows), V-C (*Rubus* stunt), and V-D (flavescence dorée) phytoplasmas have been reported in Europe (Bianco et al. 1996; Gundersen and Lee, 1996; Prince et al. 1993), and subgroup V-B (cherry lethal yellows and related phytoplasmas) has been reported in China (Lee et al. 1995). We suggest that the known lethality of elm yellows phytoplasma for American elm (*Ulmus americana* L.) (Sinclair, 1981) favors the hypothesis that, although variants of the phytoplasma may have arisen on this continent, elm yellows phytoplasma and its American elm host did not co-evolve and that an elm yellows phytoplasma was introduced into the North American elm ecosystem. It is feasible that other group 16SrV phytoplasmas also originated in geographical regions outside the range of the native North American elm, *U. americana*. In view of the apparent diversity of group 16SrV phytoplasmas on the European and Asian continents, it is possible that the epicenter of group 16SrV phytoplasma evolution is in Europe or Asia. These hypotheses and the available data make it reasonable to seek the origin of group 16SrV phytoplasmas, including flavescence dorée phytoplasma, outside of North America.

One of the important aspects of the results from this study is the finding of two species of leafhopper that may be involved in the spread of the grapevine yellows disease phytoplasmas into and within the vineyards in Israel. Similarly, the weed plants implicated as hosts of these phytoplasmas in and around the vineyards are significant. These two complementary findings target both potential insect vectors and weed plants for further study and for consideration in the design of rational means to control the continuing spread of the grapevine yellows disease in Israel. Although the ecological conditions of vineyards differ in the Mediterranean region and continental United States, wild host plants growing in and/or around the vineyards appear to be significant in the incidence of the grapevine yellows disease complex in both regions. While the group XII-A (stolbur) phytoplasma, that is apparently predominant in grapevines in Israel, has not been reported in the United States in any plant host, and the subgroup 16SrI-A phytoplasma found in grapevines in Virginia has not been reported in Israel, this study has gained evidence that grapevines are infected by group 16SrIII phytoplasma strains in both regions. Further study, will include side-by-side molecular comparisons of these group 16SrIII phytoplasma strains to determine whether or not they are mutually related. In addition, the Israel grapevine group 16SrIII phytoplasma will be compared with a group 16SrIII phytoplasma found in grapevines in Italy.

The data obtained during this project clearly indicated that varied phytoplasmas are associated with grapevine yellows disease(s) and that their identities vary with geographical region. Thus, in different regions, different insect vectors probably spread the various phytoplasmas; the different grapevine disease phytoplasmas probably have different plant host ranges; and different plant species might act as reservoirs of the various phytoplasmas.

As more data are gathered revealing the biodiversity among grapevine yellows disease pathogens, and as more information is obtained concerning their insect vectors and non-grapevine plant hosts, it is becoming clear that the grapevine yellows is a complex comprised of several diseases. While these diseases exhibit identical symptoms, they are caused by phylogenetically distinct phytoplasmas whose specific vectors, seasonal patterns of spread, and alternate host plants differ. These observations underscore the need for

precise identification, characterization, and classification of the phytoplasmas involved, and the design of disease management strategies that are tailored according to ecological situation and geographical region.

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COOPERATION

Identification by Israeli scientists of potential insect vectors of the phytoplasmas associated with grapevine yellows disease in Israel drew to the attention of the United States laboratory the need for continued surveillance and quarantine to prevent the introduction of these "exotic" insect vectors into this country. The sharing of DNA from the plants infected by the predominant grapevine yellows phytoplasma in Israel provided the opportunity to develop technology for testing the efficiency of detection of this pathogen in existing plantings and in imported germplasms in the United States. The identification by the U.S. laboratory of a group 16SrI phytoplasma in grapevines in Virginia provided the reciprocal benefit for investigation of grapevine yellows in Israel and the protection of Israeli grapevines against introduction of these pathogens.

Sequence analyses and comparisons were provided by both laboratories to each other, with base data for evaluating the molecular genetic biodiversity of phytoplasmas in grapevines, as well as other plants, and for the refinement of molecular methods to achieve a finer level of distinction among possibly different pathogen subpopulations that may differ in important aspects of epidemiology that affect disease spread and severity.

Continued exchange of materials and data improved both laboratories' ability to investigate grapevine yellows and to evaluate the impact of the disease in the two countries.

The efficient two-way transmission of materials and information between the U.S. and Israeli research groups made it possible to gain significant new data toward understanding fundamental and practical aspects of grapevine yellows in both Israel and the United States.

The results of this present cooperative study have also led to the formulation of new research questions and approaches, which are unrelated to this research project, but which will form the basis for a future joint research project that is now in the early planning stages.

MAIN ACHIEVEMENTS

Objective 1. Survey yellows disease incidence and severity in the U.S. and Israel, using as criteria yellows disease symptomatology and molecular detection of MLO (now termed phytoplasma) pathogens.

-- Incidence of grapevine yellows in vineyards in Israel and in Virginia was found to increase during the period of this study. In Israel the disease spread throughout the country during three to four years. This finding forms a basis (i) for the need to develop estimates of financial losses as well as (ii) for the need to improve management of the disease.

-- Data from spatial analysis and clustering of diseased grapevines suggested that the yellows disease was spread within vineyards by insect vector(s). This finding targeted insects as a focal point for studies to understand the disease epidemiology.

-- The pattern of disease spread in Virginia vineyards indicated that woody plants at the forest edge were the source of the grapevine infecting phytoplasmas. This work targeted plant species that were investigated as keys in the epidemiology of the disease. (Davis et al. 1997a, 1998a,b)

-- Mixed infection of yellows diseased grapevines, by phytoplasmas including the stolbur (Israel grapevine yellows) phytoplasma and a subgroup 16SrI-B phytoplasma, was revealed in grapevine yellows in northern Italy, but a search for the subgroup 16SrI-B phytoplasma in Israel and the United States gave no indication that it is present in grapevines in these latter regions. This accomplishment pointed to significant differences in the mode of spread of phytoplasmas capable of infecting cultivated grapevines. (Alma et al. 1996)

-- A reference collection of the Israel grapevine yellows phytoplasma was established, for continued and future in-depth studies and comparisons with other emerging phytoplasmas, by experimental graft transmission to *Catharanthus roseus* and molecular typing of the phytoplasma (Tanne and Orenstein 1997).

Objective 2. Identify and classify group affiliations of individual grapevine yellows associated MLO (phytoplasma) strains using DNA based molecular techniques.

-- Completed characterization of ribosomal gene sequences from two mutually distinct phytoplasmas in yellows diseased grapevines in Virginia, discovered that these two phytoplasmas have different regional distributions in Virginia, and definitively determined that the Virginia grapevine phytoplasmas are distinctly different from the phytoplasmas that cause *Flavescence doree* and *bois noir* diseases of grapevines in Europe. The findings contribute to understanding the geographic occurrence of genetically diverse phytoplasmas infecting grapevines; and to the development of an emerging taxonomy of phytoplasmas.

-- Two mutually distinct phytoplasmas were found to be associated with grapevine yellows in Virginia. One phytoplasma was identified as a tomato big bud phytoplasma (group 16SrI, subgroup A). The other phytoplasma was characterized as a previously unknown phytoplasma. This second phytoplasma was classified in a new subgroup (I) in group 16SrIII (the peach X-disease phytoplasma group). This accomplishment provided precise molecular targets (phytoplasma molecular signatures) useful for the future search for insect

vectors that are spreading the grapevine yellows disease problem in the U.S. (Davis et al. 1998a)

-- The Australian grapevine yellows disease phytoplasma was defined and characterized as a new species, "Candidatus Phytoplasma australiense", on the basis of molecular criteria, a new convention now being used to refer to lineages of prokaryotes that cannot be isolated and biologically characterized in pure culture (Davis et al. 1997a). This accomplishment established the first taxonomic recognition of a grapevine yellows disease phytoplasma, was only the second named "Candidatus Phytoplasma species", differentiated the Israel and Virginia grapevine yellows phytoplasmas from the grapevine yellows disease phytoplasma on the Australian continent, and underscored that the stolbur phytoplasma (Israel grapevine yellows phytoplasma) was probably not present in Australian grapevines, thereby refuting data published by others.

-- The phytoplasma causing grapevine yellows disease in Israel, Greece, and southern Italy was identified and molecularly characterized as a member of group 16SrXII, subgroup A (the stolbur phytoplasma subgroup). This accomplishment provided the first definitive, thorough characterization of the grapevine phytoplasmas in Israel, southern Italy, and Greece, providing evidence for the widespread occurrence of this grapevine yellows phytoplasma. (Albanese et al. 1996; Davis et al. 1997c; Tanne and Orenstein 1997).

-- Determination that grapevines suffering from yellows disease in some regions of eastern Europe are infected by the same phytoplasma (stolbur phytoplasma) that predominates in diseased grapevines in Israel. This has been known to cause diseases in vegetable crops including tomato and pepper, and its wide geographical distribution suggests it could be of increasing concern in several important crops including grapevine. (Kolber et al. 1997)

Objective 3. Design polymerase chain reactions for specific detection and identification of individual grapevine yellows associated MLO (phytoplasma) strains or groups of strains for detailed analysis of the epidemiology of the disease.

-- The oligonucleotide primers previously designed by others for the specific detection and identification of the stolbur phytoplasma (cause of grapevine Bois Noir disease, and grapevine yellows in Israel, Greece, and elsewhere in the Mediterranean region) were found to be non-specific for this phytoplasma. This project discovered these oligonucleotides to prime amplification of DNA also from other phytoplasmas, including the Australian grapevine yellows phytoplasma ("Candidatus Phytoplasma australiense"), Mexican periwinkle virescence phytoplasma, and a phytoplasma discovered in this work to be involved in an emerging strawberry disease complex in Florida. The results indicated that the existing primers were of limited, and possibly misleading, usefulness in epidemiological studies. (Davis et al. 1997a ; Jomantiene et al. 1996, 1998).

-- A portion of the ribosomal protein gene operon of the Israel grapevine yellows phytoplasma (stolbur phytoplasma) was amplified in PCR and its nucleotide sequence was determined. Sequence alignments with comparable DNA molecules from other phytoplasmas indicate regions of potential use in the design of new, specific primers.

Objective 4. Discover and identify potential insect vectors and alternate plant hosts of grapevine yellows associated MLOs (phytoplasmas).

-- Alternate, natural host of phytoplasma affecting commercial grapevines in Virginia was identified as Vitis riparia (wild grapevine) through the specific amplification in PCR of

phytoplasmal DNA and its RFLP and nucleotide sequence analyses. The infected V. riparia plants were symptomless. This accomplishment targeted this wild plant as a location where insect vectors of the grapevine yellows phytoplasma may be found; and it points to tolerance of the phytoplasma in a Vitis species, thereby opening new avenues for future disease management and control. (Davis et al. 1998b).

-- Hyalesthes obsoletus and Neoaliturus guttulatus were identified as potential leafhopper vectors responsible for the spread of the grapevine yellows phytoplasmas in Israel. This accomplishment targeted insect vector species for possible future intervention in the transmission cycle of the Israel grapevine yellows disease complex.

Objective 5. Test the efficacy of the antibiotic oxytetracycline for the remission of disease symptoms and the reduction of MLO (phytoplasma) infection in grapevine. (This objective was modified because of legal restrictions related to antibiotic usage. Thus, study of the effect of mechanical barriers to reduce disease spread was initiated.)

-- Work was initiated to test the efficiency of agrotechnical methods to reduce disease spread. The experiment is planned as a long term trial and will continue for the next 3-4 years. The outcome of this experiment could lead to new approaches to manage grapevine yellows disease in and other Mediterranean regions.

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TABLES AND FIGURES

Table 1.

Cultivar	Location	Viticultural area	PCR (Universal)
Chardonnay	North-Israel	Golan	+
Cabernet sauvignon	North-Israel	Golan	+
Sauvignon blanc	North-Israel	Golan	+
Merlot	North-Israel	Golan	+
Gwurtztraminer	North-Israel	Golan	-
Riesling	North-Israel	Golan	-
Cabernet sauvignon	North-Israel	Galil	+
Chardonnay	North-Israel	Galil	+
French colombard	North-Israel	Golan	+
Chardonnay	Center-Israel	Samaria	+
Carignan	North-Israel	Galil	+
Chardonnay	Center-Israel	Shfela	+
Carignan	Center-Israel	Shfela	+
Cabernet sauvignon	Center-Israel	Shfela	+
Cabernet sauvignon	Center-Israel	Shfela	-
French colombard	Center-Israel	Shfela	+
Merlot	Center-Israel	Shfela	+
Chardonnay	Center-Israel	Shfela	+
Perlet	Center-Israel	Shfela	-
Superior	Center-Israel	Shfela	-
Chardonnay	South-Israel	Arad	+
Cabernet sauvignon	South-Israel	Arad	+
Merlot	South-Israel	Arad	+
Sauvignon blanc	South-Israel	Arad	-
Zeini (table grape)	South-Israel	Arad	+

PCR analysis demonstrated the presence of phytoplasma in many wine cultivars in all grapevine growing regions in Israel. The disease spread in 3-4 years all over the country. We could not detect phytoplasma in plants that did not exhibit symptoms. In one case phytoplasma was detected in a table-grape variety growing close to a Chardonnay vineyard.

Table 2: Location and Trapping Time of Leafhoppers and Planthoppers in Israeli Vineyards

Plant/leaf-hopper	Location	Time	PCR analysis for phytoplasma
<i>N. guttulatus</i>	North-Golan	June 1997	-
<i>N. guttulatus</i>	Center-Golan	June 1997	+
<i>Macrosteles</i>	North-Golan	June 1997	-
<i>Macrosteles</i>	Center-Golan	June 1997	-
<i>N. guttulatus</i>	North-Golan	July 1997	+
<i>N. guttulatus</i>	Center Golan	July 1997	N.T.
<i>H. obsoletus</i>	Center-Israel	July 1997	-
<i>N. guttulatus</i>	Center-Israel	July 1997	+
<i>N. guttulatus</i>	North-Golan	August 1997	+
<i>N. guttulatus</i>	Center-Golan	August 1997	+
<i>Macrosteles spp.</i>	North- Golan	August 1997	-
<i>Macrosteles spp.</i>	Center-Golan	August 1997	+
<i>N. guttulatus</i>	North-Golan	September 1997	+
<i>N. guttulatus</i>	Center-Golan	September 1997	N.T.
<i>H. obsoletus</i>	North-Golan	September 1997	+
<i>H. obsoletus</i>	Center-Golan	September 1997	+
<i>H. obsoletus</i>	Center-Israel	September 1997	+
<i>Macrosteles spp.</i>	North-Golan	September 1997	N.T.
<i>Macrosteles spp.</i>	Center-Golan	September 1997	N.T.
<i>H. obsoletus</i>	North-Golan	October 1997	+
<i>H. obsoletus</i>	Center-Golan	October 1997	+
<i>H. obsoletus</i>	Center-Golan	November 1997	+
<i>N. guttulatus</i>	North-Golan	November 1997	-
<i>N. guttulatus</i>	Center-Golan	November 1997	+

Spatial-Dispersion of Yellows in Vines
 Quadrat-Variance Method

Spatial-Dispersion of Yellows in Vines
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Fig 2: Detaction of phytoplasma by PCR
analysis of symptomatic grapevines.

From left: -: negative control. M: | Hind III. Lane 1: DNA from healthy grapevine. Lane 2: Chardonnay-central country. Lane 3: Carignane-central country. Lane 4: Chardonnay-southern country. Lane 5: Cabernet-southern country. Lane 6: Chardonnay-northern country. Lane 7: pCX. Lane 8: pEY. Lane 9: pAY. Lane 10: Chardonnay-southern country. Lane 11: Chardonnay-northern country. Lane 12: positive control.

Fig 3: Determination of phytoplasma type by nested PCR with group specific primers.

AY; Aster Yellow. CX; Canadian Western X. From left: Lane 1: Chardonnay-northern country. Lane 2: Cabernet-northern country. Lane 3: Carignane- central country. Lane M: pBR *May* I. Lane 4: Cabernet -southern country. Lane 5: Chardonnay-southern country. Lane 6: Carignane- northern country. Lane +: positive control.

Fig 2: Determination of phytoplasma type by *Mse* I restriction analysis of the nested products.

From left: Lane M: pBR *Alu* I. Lane CX: positive Canadian Western X control. Lane 1: Chardonnay-northern country. Lane 2: Cabernet-northern country. Lane AY: positive Aster-Yellows control. Lane 1: Chardonnay-northern country. Lane 2: Cabernet-northern country. Lane 3: Carignane-central country. Lane 4: Cabernet-southern country. Lane 5: Chardonnay-southern country. Lane 6: Carignane-northern country. Lane M: pBR *Alu* I.

Fig 3: Determination of phytoplasma type by *Kpn* I restriction analysis of the nested products.

From left: Lane CX: positive Canadian Western X control. Lane 1: Chardonnay-northern country. Lane 2: Cabernet-northern country. Lane AY: positive Aster-Yellows control. Lane 1: Chardonnay-northern country. Lane 2: Cabernet-northern country. Lane 3: Carignane-central country. Lane 4: Cabernet-southern country. Lane 5: Chardonnay-southern country. Lane 6: Carignane-northern country.

Fig 6: Detection of phytoplasma in leafhoppers and planthoppers by PCR using Universal Primers.

The DNA template in each lane amide with 5 particular leaf or planthoppers.

Lane M: pBR Mav I. Lane 1: *Circulifer spp.* Lane 2: unidentified. Lane 3: *H. Obsolatus*. Lane 4: unidentified. Lane 5: *Circulifer spp.* Lane 6: *H. Obsolatus*. Lane 7: negative control. Lane 8: positive control. Lane 9: *Circulifer spp.* Lane 10-11: *N. guttulatus*. Lane 12: *H. Obsolatus*.