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Distribution of phytoplasma in grapevines in the Golan Heights, Israel, and development of a new universal primer

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Summary

Our survey, made in 1999 and 2000, sampled 4 of the most prevalent grapevine cultivars in northern, central and southern regions of the Golan Heights. There were significant differences in the levels of phytoplasma infection between different sub-regions of the Golan Heights; highest levels were found in the south, followed by the center, and the lowest levels in the north. Stolbur was found to be the predominant phytoplasma (~70 %), although aster yellow (~11 %), western-X (~5 %) and mixtures of two phytoplasmas (13 % of all infections, 90 % of which involved Stol and AY) were also found. Prior research on phytoplasmas of grapevines in Israel involved the use of two sets of universal primers. Primers were developed specifically for phytoplasmas occurring in Israel in which there were homologies of 90, 85 and 78 % for Stol, AY and W-X phytoplasmas, respectively.

K e y w o r d s : phytoplasma, stolbur, aster yellows, western-X, diagnosis, PCR, RFLP.

Introduction

Phytoplasmas can cause yellows disease in plants. The symptoms of yellows diseases in grapevine are delayed budburst, yellowing or reddening of leaves, backward curling of leaves, immature canes and poor set or shrivelling of berries. It is not clear whether the symptoms are due to the presence of the phytoplasma itself or whether they appear as a result of the toxins or alterations in plant hormones mediated by phytoplasma (CHEN and CHEN 1998). However, in most cases, symptoms indicate the presence of phytoplasma and vice versa.

There are limitations to molecular analysis and differences exist between the molecular analysis of an old leaf with symptoms and a young leaf without clear symptoms. Old phytoplasma-infected grape leaves are brittle and have high levels of phenols that can interfere with PCR. Furthermore, it is well known that heat shock contributes to the disappearance of phytoplasma particles from foliage, stem and root areas. Plants with clear symptoms, which were exposed to temperatures $\geq$ 40 °C for varying lengths of time, were cured of phytoplasma infection (CAUDWELL *et al.* 1997).

In grapevine, phytoplasmas belonging to 5 subgroups are known to occur worldwide: stolbur (Stol) phytoplasma in northern France (DAIRE *et al.* 1997 a), Germany (MAIXNER *et al.* 1995), Israel (DAIRE *et al.* 1997 a), Italy (MARCONE *et al.* 1996), Spain (LAVINA *et al.* 1995) and Switzerland (DAIRE *et al.* 1997 a); elm yellows (EY) phytoplasma in France (DAIRE *et al.* 1997 a), Germany (DAIRE *et al.* 1997b) and Italy (PRINCE *et al.* 1993; DAIRE *et al.* 1997 a); aster yellows (AY) in Australia (GIBB *et al.* 1999; CONSTABLE *et al.* 2000), Germany (PRINCE *et al.* 1993), Israel (TANNE and ORENSTEIN 1997) and Italy (BIANCO *et al.* 1996); western-X (W-X) phytoplasma in Israel (TANNE and ORENSTEIN 1997), Italy (BIANCO *et al.* 1996) and USA (PRINCE *et al.* 1993), and *Candidatus* Phytoplasma *australiense* in Australia (DAVIS *et al.* 1997). It is known that a single grapevine can have two or more phytoplasma infections and that these can be from the same subtype or from different subtypes (ALMA *et al.* 1996). Furthermore, different grape cultivars have different levels of phytoplasma infections (REFATTI *et al.* 1991), Chardonnay being the most susceptible. Some information is available on the distribution of phytoplasmas in Europe; however, the distribution and percentage of each type is unknown for grapevines in Israel.

The Golan Heights in Israel is a comparatively new, but economically important, wine growing area. The topography over the 50 km length of the Golan Heights ranges from an elevation of 1000 m in the north to 300 m in the south. Soil in the Golan Heights is of volcanic origin. Winter and summer temperatures vary greatly between the northern and southern borders. This paper reports on the development of specific primers for the phytoplasmas found in grapevines in Israel and the distribution of different phytoplasma types in different cultivars and regions of the Golan Heights.

Material and Methods

L e a f s a m p l e s (Tab. 1): A survey was carried out in 1999 in three sub-regions of the Golan Heights: Odem and El-Rom in the north, elevation 1000 m; Alonai HaBashan and Yonatan in the center, elevation 600-700 m; and G'shur in the south, elevation 350 m. Samples were collected just before harvest (last week in August till the first week in September) when symptoms were clearest.

To be scored positive for phytoplasma infection, a vine had to exhibit at least two of the following symptoms: yellowing or reddening of leaves, backward curling of leaves, immature canes, poor fruit set, shrivelling of berries. Symp-

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Table 1

Survey of grapevines with signs and symptoms of phytoplasma infection

Place	Variety	Total number of vines examined	Number of signs and symptoms	Percent infection[1]
Northern Golan Heights				
Odem	Merlot	1500	3	0.002
	Chardonnay	1095	291	26.6
El-Rom	Sauvignon blanc	2944	66	2.2 a
	Cabernet Sauvignon	2208	8	0.4 A
Central Golan Heights				
Alonai HaBashan	Merlot	1380	90	6.5
	Cabernet Sauvignon	1428	228	15.9 B
Yonatan	Sauvignon blanc	2100	388	16.1 b
	Chardonnay	2100	1464	69.7
Southern Golan Heights				
G'shur	Merlot	10[2]	4	
	Sauvignon blanc	2040	499	24.5 c
	Cabernet Sauvignon	1530	389	25.4 C
	Chardonnay	15[2]	10	

[1] Percent infection followed by different upper or lower case letters were highly significantly different ($P << 0.001$).
[2] Small experimental plot.

tomatic grape leaves from 4 varieties (Cabernet Sauvignon, Merlot, Sauvignon blanc, Chardonnay) were collected. Generally 20 infected grapevines were randomly selected for sampling. In cases where less than 20 were sampled, these represent the total number of infected plants in the vineyard. About 5 symptomatic leaves (the youngest or apical leaves on a shoot) were collected from each plant. Since phytoplasmas are more concentrated in the main veins, tissue between the veins was removed before leaves were stored at -20°C.

Data were analyzed with CoStat Statistical Software (Minnesota, MN, USA) by 1-way analysis of variance (ANOVA) after arcsine transformation of percentage of infection data. Means were separated by Tukey-Kramer multiple range test. All tests were conducted at $\alpha = 0.05$ level.

DNA extraction: Total DNA was extracted from plant tissue as described by MAIXNER *et al.* (1995). Plant tissue (1 g) was homogenized in a total of 8 ml; 3 % cetyl trimethyl ammonium bromide (CTAB), 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.2 % ß-mercaptoethanol. After centrifugation (8,000 x *g*) the supernatant was mixed with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and recentrifuged (8,000 x *g*). The supernatant was mixed with an equal volume of isopropanol and centrifuged (20,000 x *g*). The extracted DNA was washed once with 70 % EtOH, then resuspended in 100 µl dH_2O, and stored at -20 °C.

DNA amplification and PCR analysis: Polymerase chain reaction (PCR) amplification of DNA was performed using a Tgradient thermocycler (Tamar Laboratory Supplies, Israel). The purified DNA was passed through two cycles of PCR – the first in the presence of universal primers defined as P1 and P7 (DENG and HIRUKI 1991) and the second in the presence of universal primers defined as rU3 and fU5 (Lorenz *et al.* 1995). Amplification was carried out in a total of 30 µl; 0.3 µl (5 units/µl) *Taq* polymerase (Promega), 3 µl amplification buffer without $MgCl_2$, 2 µl 25 mM $MgCl_2$, 3 µl 2 mM DNTPs, 0.5 µl 50 pM each primer, and 5-10 ng DNA. The reaction mixture was incubated at 94 °C (5 min), 50 °C (2 min), 72 °C (2 min); 35 cycles of 92 °C (45 s), 50 °C (45 s), 72 °C (90 s). The last cycle (72 °C) was extended for 7 min as an elongation step. The amplified PCR products were analyzed by electrophoresis of 12 µl of reaction mixture in 1.5 % agarose gel (40 mM Tris-HCl, pH 7.5; 20 mM acetic acid; 1 mM EDTA), stained with ethidium bromide and visualized with a UV transilluminator. The size standard 100 bp DNA ladder used in gels was obtained from MBI Fermentas. DNA extracted from asymptomatic plants served as a negative control.

Restriction pattern analysis: In order to classify the phytoplasma, each positive PCR product was analyzed separately in a RFLP reaction in the presence of restriction enzymes *Tru9* I and *Alu* I (Promega). DNA of each phytoplasma type, previously obtained from E. BOUDON-PADIEU in France, were used as standards. The restriction products were then separated by electrophoresis through a 5 % polyacrylamide gel and stained with ethidium bromide and visualized with a UV transilluminator.

Data were analyzed with SAS Institute Inc. software (Cary, N.C., U.S.A.) by χ^2 analysis. Means were separated by Fisher's Exact Test (2-Tail). Statistical comparisons were made between levels of phytoplasma infections in red and white varieties. All tests were conducted at $\alpha = 0.05$ level.

C l o n i n g o f P C R p r o d u c t s : PCR products characterizing 4 restriction profiles of phytoplasma from the AY, W-X, Stol groups and the profile characteristic of combined infection were cloned using the TA-cloning method (Invitrogene). The cloning products were sent to M. KORNER for sequence analysis at the sequence identification unit, Hebrew University, Givat Ram, Israel. The products were analyzed for the degree of homology with data deposited in GenBank. The sequence analysis products were processed using ASSEMBLY software and MC VECTOR software in order to detect specific universal primers. The primers were tested using BLAST in order to identify the most universal pair for phytoplasma detection.

Results

P h y t o p l a s m a d i s t r i b u t i o n : A survey of the percentage of grapevines infected with phytoplasmas was conducted in 1999 and results are shown in Tab. 1. Two varieties are planted in all three sub-regions, Cabernet Sauvignon and Sauvignon blanc. There were highly significant differences ($P < 0.001$) in the levels of phytoplasma infections for both varieties in the three regions; highest levels were found in the south, followed by the central region, and lowest infection levels were found in the north.

The results of the PCR analysis of symptomatic plants (Tab. 2) show that positive PCR results from plants with characteristic symptoms of phytoplasma infection ranged from 0 to 70 %. In order to determine the phytoplasma group distribution in different vineyards, the PCR products of infected plants were analyzed using a RFLP reaction (Tab. 2). Since there were only a few infections with AY, W-X or a mixture of two phytoplasmas, their distribution could not be analyzed. However, χ^2 analysis of the number of Stol infections versus all others could be performed. In red varieties, there were significantly less ($P < 0.05$) Stol infections in the center of the Golan Heights (*i.e.*, more AY, W-X and mixed infections) than in the northern or southern regions. For white varieties, there was no significant difference in the number of Stol infections versus other phytoplasma groups among the different regions of the Golan Heights (*i.e.*, there was just as much AY, W-X or mixed phytoplasma infection in white varieties in all regions).

C l o n i n g , c h a r a c t e r i z a t i o n a n d d e t e c t i o n o f s p e c i f i c p r i m e r s : Primers from the 5' region of the 16S rDNA gene were chosen. The length of their PCR product is 174 bp. Their notation is IU-r2 and IU-f1 and the sequences are shown in Tab. 3. The previously used nested primers produced PCR products of 850 pb.

The Figure represents a comparison between the two analytical methods using universal primers. The new primers required only a single PCR reaction to obtain the products; hence the bands in lanes 1-4 appear lighter than those from the nested products (lanes 5-8). In the previous analysis, two PCR reactions were required to obtain the PCR product, the second reaction amplifying the products of the first reaction.

Discussion

We found a distinct gradient in the rate of phytoplasma infections with significantly lower levels in the northern, cooler sub-region, as compared to the southern, distinctly warmer, sub-region. The number of specific types of phytoplasma (Stol, AY and W-X) was too small to be analyzed by sub-region.

T a b l e 2

Grapevines with signs and symptoms of phytoplasma infection and phytoplasma type

Place	Variety	Number of plants sampled	Number of PCR positive samples	Number of vines infected[1] with:			
				Stol	AY	W-X	Mixed infection
Northern Golan Heights							
Odem	Merlot	3	0				
	Chardonnay	20	0				
El-Rom	Sauvignon blanc	13	8	5	2	1	
	Cabernet Sauvignon	14	11	10	1		
Central Golan Heights							
Alonai HaBashan	Merlot	20	9	5	1	1	2 (Stol and AY)
	Cabernet Sauvignon	20	15	9	3	2	1 (Stol and AY)
Yonatan	Sauvignon blanc	20	3	2			1 (AY and W-X)
	Chardonnay	10	8	6			2 (Stol and AY)
Southern Golan Heights							
G'shur	Merlot	4	0				
	Sauvignon blanc	20	14	11	2		1 (Stol and AY)
	Cabernet Sauvignon	20	9	9			
	Chardonnay	10	5	1			4 (Stol and AY)

[1] Based on RFLP analysis.

Table 3

Sequences of the primers

IU-f1:	5'AAT GGA GGA AAC TCT GAC CG 3'
IU-r2:	5'AAC GCT TGG CCC CTA TGT ATT ACC 3'

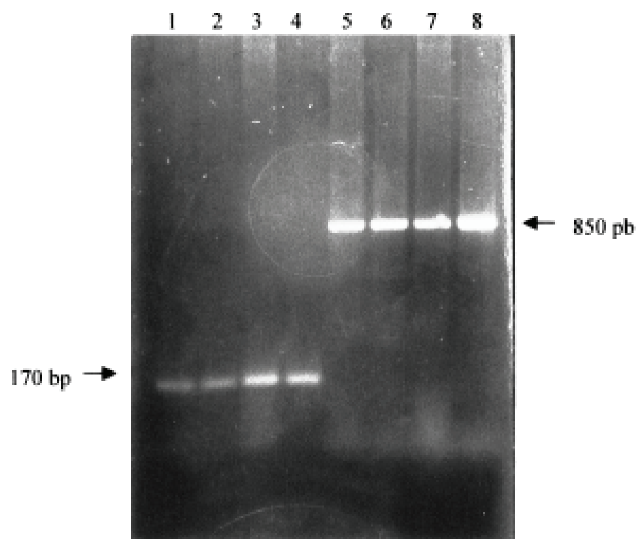

Figure: PCR products obtained with primer pair IU-r2 and IU-f1 (lanes 1-4) and nested PCR products obtained with primer pairs P1 & P7 and rU3 & fU5. Lanes 1 and 5, aster yellows; lanes 2 and 6, western-X; lanes 3 and 7, elm yellows; lanes 4 and 8, Stol.

The results of the survey indicate that stolbur phytoplasma is the most widespread type in the Golan Heights, about 70 % of single phytoplasma-infected grapevines being infected with it. This form of Stol phytoplasma is different from the one commonly found in Europe based on the sequence analysis of data deposited in GenBank. Statistically, there was no difference in the level of Stol infections in white varieties among all regions of the Golan Heights. There were significantly fewer Stol infections in red varieties (more AY, W-X and mixed phytoplasmas) in the central region versus the north and south. Notably, Stol phytoplasma is also the most common in northern France, Germany and Italy.

The second most prevalent type of phytoplasma was aster yellows; about 11 % of infected plants with a single-phytoplasma infection were infected with AY. An AY is also reported in grapevines in Australia (GIBB *et al.* 1999; CONSTABLE *et al*. 2000). Previous work examining infected grape samples from all over Israel indicated that AY was the predominant type (ORENSTEIN 1999).

Approximately 5 % of plants were infected with a unique profile; the restriction profile obtained by the RFLP analysis indicates a relation to the Western-X group of phytoplasmas. This group of phytoplasmas was first detected in vineyards in Israel in 1996 (ORENSTEIN 1999) and is not common to other vineyards in the world. It is also not common in other regions of Israel.

Approximately 13 % of infected plants showed dual phytoplasma infections within the same plant. It is unclear whether the characteristic symptoms for single phytoplasma are aggravated as a result of a combined infection. Previous results showed a significant negative correlation between infection with AY phytoplasma and W-X phytoplasma (ORENSTEIN 1999). This result suggests competition between these strains of phytoplasma for plant resources. Indeed, in this survey only one plant with a dual infection was found to have both AY and W-X; of the remaining grapevines with dual infections, all were stolbur and AY except one, which was stolbur and W-X. It is unknown if the presence of one strain of phytoplasma suppresses another strain. The relationship between phytoplasmas in a dual infection is not clear because biotic and abiotic factors are involved.

The cost of identifying the phytoplasmas in plants showing signs and symptoms of infection was about 7 US$ per sample. In order to decrease these costs, new universal primers were selected. Three PCR products, representing different groups of phytoplasmas as determined by the survey performed in the Golan Heights, were cloned using the AT-cloning method. The cloning products were sent for sequence analysis. GenBank data were used to determine the degree of homology of these sequences to groups of phytoplasmas found in the restriction profile. Sequence analysis of three cloning products from the stol, AY, and W-X groups showed a homology percentage of 90, 85, and 78 %, respectively. Thus, the phytoplasmas found in Israel belong to these groups but are also specific to Israel. These primers were found to be efficient and reliable. Only a single PCR reaction was required to determine phytoplasma infection in suspected plants; therefore, the cost of analysis was substantially reduced. The new PCR product was only 174 bp, so the reactions also were faster.

Even with these new primers, PCR analysis of a number of vines with symptoms of phytoplasma infection revealed no amplification product. Since the survey was conducted at the end of the season, immediately before harvest, there could have been interference from accumulated salts, heavy metals, plant phenols etc. known to adversely affect PCR reactions. Furthermore, heat shock could also have been a factor since daytime temperatures rose above 35 °C in summer before leaves were harvested. CHEN and CHEN (1998) have evidence that symptoms may be a result of toxins or alterations in plant hormones mediated by phytoplasmas. More research is needed to elucidate the factors involved in this situation.

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