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Characterization of the yeast flora on the surface of grape berries in Israel

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Summary

Yeast populations were collected from the surface of berries of three grape cultivars during three seasons, from fruit set to maturity. They were studied by RAPD and ap-PCR, each with two primer pairs. In the population, identical isolates were found only rarely on 13 % of the bunches in 1997 and on 58 % of the berries in 1999. From RAPD and ap-PCR, a dendrogram with clusters of similarity was established. Eleven representatives from clusters of the white yeast dendrogram were identified by traditional methods as 10 different yeast species, one of which has not been isolated from grape berry surfaces before. The population size was smaller for Colombard than for Cabernet Sauvignon and Muscat of Alexandria berries.

Key words: berry, epiphytic flora, RAPD, PCR.

Introduction

Yeast and yeast-like organisms are part of the natural flora on plant surfaces (Belin 1972; Droby *et al.* 1999; Pretorius 1999), they are part of the epiphytic balance and a change in their occurrence may affect disease development. Chalutz *et al.* (1989) have shown that water-washed citrus fruit decayed more quickly than unwashed fruit, possibly due to the removal of the natural epiphytic flora. As a consequence pathologists began to search for fungi, bacteria and yeast strains, to be used as biological control agents on different crops. When searching for a biological control agent, it is advisable to start with organisms that are native to the plant since they will be well adapted to the crop and will have a good chance to survive if applied as augmentation sprays. It is important to screen a large number of isolates in order to locate types that appear repeatedly and for this a reliable screening method is needed.

The cost of classic yeast identification is high and as a consequence, in ecological studies where many isolates are involved, usually only morphologically distinctive types are chosen as representatives for ultimate identification. With such a screening method, isolates that look alike but are actually different might therefore be overlooked. On the other hand, identifying the isolate by name is not enough if properties such as antagonistic activity are important. Different strains of the same yeast species may differ in their antagonistic activity (Filonow *et al.* 1996; Schena *et al.* 1999).

RAPD-PCR does not require preliminary knowledge concerning the genome of the tested organism. This method can be used to screen a large number of strains and to recognize if there are types that are more abundant and therefore probably more adapted to the crop in question. Besides, such surveys facilitate choosing strains that are genetically different and so enable obtaining a comparatively wide range of different species and isolates that form the natural yeast flora. A good screening method can help to detect different strains in the population, even if they represent a comparatively small part of the microflora. Such a method also eliminates the possibility of working with identical strains from a selected population.

Most studies on the natural flora on grapes and in grape musts have dealt with the flora of mature grapes and with changes in yeast populations during fermentation. Very little work has been done on the flora of grapes during berry development. The aims of this work were to characterize the yeast flora on berries of three cultivars from fruit set to maturity using molecular methods, to identify strains that differ distinctly and to select strains that are more abundant than others with the ultimate objective of selecting suitable candidates for biological control of fungal pathogens.

Material and Methods

Collection of yeast strains: Yeast strains were collected during the growing seasons of 1995, 1997 and 1999. In 1995 and 1997 strains were collected in an experimental breeding vineyard at the Volcani Center, located in the Coastal Plain of Israel. Grape berries were sampled from cvs Cabernet Sauvignon, Muscat of Alexandria and Colombard. All plants had the same canopy management and received the same chemical sprays. Samples were collected every two weeks, from two weeks after fruit set (April) until fruit maturity. Each time, three bunches (=replicates) were sampled for each cultivar and from each sample 25-50 berries (depending on berry size) were collected aseptically in sterile cups. The berries were weighed and sterile distilled water proportional to the berry volume was added to enable calculation of the number of CFU per unit berry surface area. In 1999 sampling was slightly different from that of previous years. Samples were collected 4 times at monthly intervals from a commercial vineyard on the Golan Heights. Each time 4 bunches were sampled from each cultivar by picking 4 berries per bunch and immersing them individually in cups with sterile distilled water. The cups with the berries were then shaken on a horizontal shaker for 1 h at 150 rpm. The wash water was serially diluted and 3 replicates (30 µl) of each dilution were plated in Petri dishes (one per replicate) with basal yeast agar (BYA, containing 20 g glucose, 1 g yeast extract, 10 g protease pep-

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tone and 15 g agar in 1 l and amended with 250 mg Penicillin G to suppress growth of bacteria). The number of yeast colonies that developed was counted after 3-5 d at room temperature. The surface area of the berries was calculated, from berry volume assuming a spherical shape. Yeast density (n) was calculated and a Log_{10} transformation of (n+1) was used to calculate variance and means that were compared by SAS PROCEDURE GLM and Duncan's test (SAS Institute, Cary, NC, USA).

Selected yeast colonies were chosen based on colour and morphological colony characteristics for further analysis. Each selected colony evolved from a single cell and was therefore considered a different strain. They were removed with a sterile plastic loop to fresh BYA plates to obtain pure cultures which were held at 4-5 °C. When possible, two colonies with the same morphological characteristics were sampled from each plate (same bunch or berry source).

D N A e x t r a c t i o n : DNA was extracted according to Hoffman and Winston (1987) with slight modifications. Yeast cells were taken from pure cultures, suspended in a 1.5 ml microtube containing 100 µl breaking buffer (2 µl Triton x-100, 1 µg SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8) and 1 mM EDTA) and vortexed briefly. Approximately 100 µl of acid-washed glass beads (Ø 425-600 ìm, Sigma-Aldrich, Milan, Italy) and 100 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added. The tubes were vortexed at high speed for 5 min followed by centrifugation for 5 min at 14,000 rpm. The supernatant was used as a template for PCR reactions.

R A P D a n d a r b i t a r y p r i m e d P C R : RAPD reaction was performed by using one of two primers (OpC-5-5' GATGACCGCC and OpD-20 - 5' ACCCGGTCAC). The reaction included 0.5 µl of the supernatant from the DNA extraction tube as a template, 50 mM KCl, 10 mM Tris-HCl, 100 µM of each of the dNTPs, 2 mM $MgCl_2$, 0.5 unit *Taq* polymerase (Dinazyme, Finnzymes OY, Finland) and 5 pmol of the primer in 25 µl final volume. The reaction tubes were incubated in a thermocycler (PTC-100, Peltier-effect Cycling, MJ Research, INC, USA) starting with 5 min of denaturation at 95 °C, followed by 30 cycles consisting of 30 s at 95 ^{0}C, 30 s at 48 °C and 1.5 min at 72 °C, the last cycle ending with 10 min at 72 °C. Arbitrary primed (ap) PCR was conducted using one of two primers derived from microsatellite sequences: $(GACAC)_3$ and $(CAG)_5$ (Freeman and Katan 1997). Reaction mixture included 50 mM KCl, 10 mM Tris-HCl, 0.2 mM of each of the dNTPs, 1.5 mM $MgCl_2$, 1 unit *Taq* polymerase, 1 µM of one of the two primers and 0.5 µl of the supernatant from the DNA extraction tube. Final reaction volume was 25 µl.

Amplification products of PCR reactions were separated in 2 % agarose gels in Tris-acetate-EDTA buffer by electrophoresis at 100 V for 20 min.

M o l e c u l a r c o m p a r i s o n o f y e a s t i s o l a t e s : For the 1997 collection the initial screening was done with two RAPD and two ap-PCR primers; it included all the isolates collected on a certain date (usually 15-30). They were run together, with the whole procedure (DNA extraction, PCR reactions and electrophoresis) repeated twice with each of the 4 primers. In the next step clearly identical isolates were left out and the remaining strains were divided according to colony color into 4 groups (white, orange, pink and beige). DNA of the isolates in each group was extracted again and they were compared using RAPD and ap-PCR. Electrophoretic fragments, obtained separately with each primer, were arbitrarily labeled and their presence or absence in each of the isolates was recorded. A data set was constructed consisting of all the arbitrary labeled fragments and their presence or absence in each isolate. Nei's resembling function (Nei and Li 1985) was used to classify and separate clusters of strains with different degrees of similarity. Results are expressed as a dendrogram, using similarity values to create clusters of strains.

The isolates collected in 1999 were analyzed using two RAPD primers (OpC-5 and OpD-20). Electrophoretic patterns of strains isolated from the same berry were compared to find the percentage of berries supporting a number of identical strains.

I d e n t i f i c a t i o n o f i s o l a t e s : Selected yeast isolates were identified by the Centraalbureau voor Schimmeelcultures (Baarn, The Netherlands) based on colony and cell morphology, fermentation abilities, growth on different C and N compounds, sensitivity to cyclohexamide and growth at 37 and 40 °C.

Results

T o t a l y e a s t p o p u l a t i o n d y n a m i c s d u r i n g t h e g r o w i n g s e a s o n : The dynamics of the total yeast population on the three grape cultivars during three years are presented in Fig. 1. The trends in the fluctuation of yeast populations on berries of the three cultivars tended to be in the same direction (e.g. the increases in June 1995 and from the end of July 1997). This may be a result of weather conditions or of a pesticide that was applied in the vineyard. Each year the average yeast population over the season was highest for Cabernet Sauvignon grapes (significant in 1999) and lowest for Colombard (Fig. 1 B), in spite of the differences in the absolute annual values and seasonal fluctuations. Populations on Colombard berries were lowest over the three years for 11 of the 18 sampling dates, and significantly higher for Muscat of Alexandria and Cabernet Sauvignon berries for 3 and 4 of the dates respectively (Fig. 1A).

M o l e c u l a r p r o f i l e s : On 8 dates during the 1997 season a total of 183 yeasts was isolated and their RAPD profiles were compared. The profiles obtained were very diverse and only in 8 cases, patterns of two or more isolates collected on the same date, were identical with the 4 primers used. Fig. 2 is a sample of a gel comprising the isolates that were collected on one sampling date (August 10). While isolates 605 and 608 look identical with primer OpD-20 (Fig. 2 B), they have different electrophoretic patterns with primer OpC-5 (Fig. 2 A) and are therefore considered different strains.

After screening identical isolates at each sampling date, 117 yeast isolates were grouped according to color (24 white, 26 pink, 33 orange and 34 beige) and isolates in each group were compared. In three cases only, two isolates collected

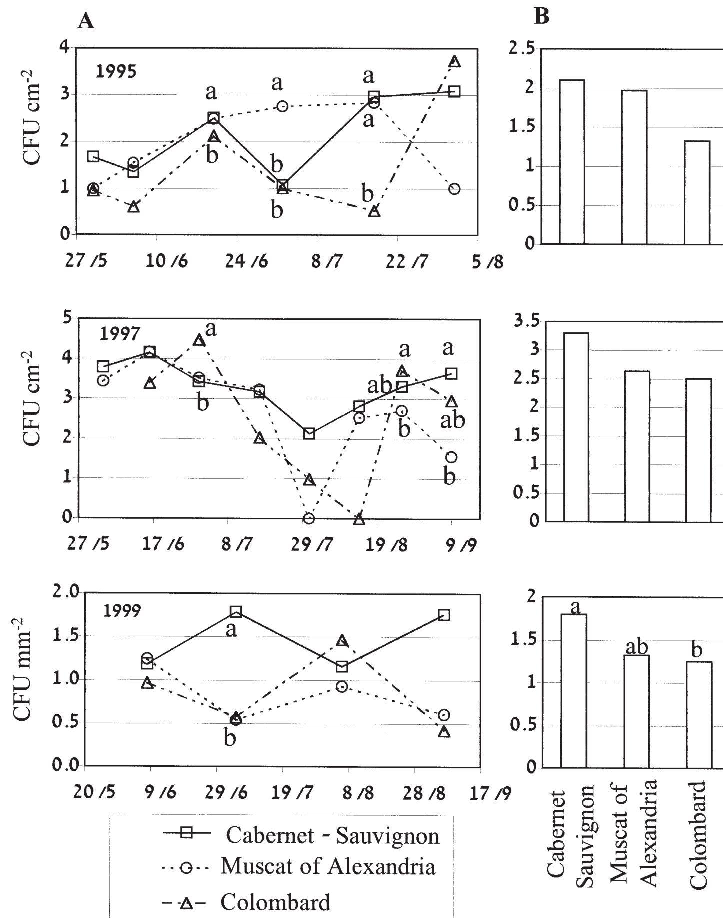

Fig. 1: Fluctuations in total yeast populations during a three-year survey on grape berries of three cultivars – Cabernet Sauvignon, Muscat of Alexandria and Colombard (**A**) and seasonal average of CFU for each cultivar (**B**). Different letters indicate significant differences at each sampling date ($p<0.05$) (A) or seasonal differences (B) between cultivars.

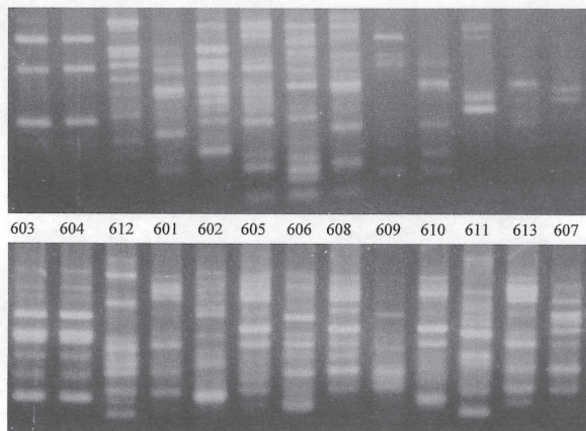

Fig. 2: Band patterns of RAPD-PCR amplification of genomic DNA of yeast isolates obtained with two primers: Op-C5 (**A**) and Op-D20 (**B**). - Isolates 601-602 were isolated from one Colombard cluster and isolates 603-606 from another; 607-609, 611-612 and 610 originated from three Cabernet Sauvignon clusters and 613 from a cluster of Muscat of Alexandria, all collected on the same date in 1997.

on different dates, had the same DNA profiles with the 4 primers used. Analysis of the data by creating a dendrogram showed that variability was greatest within the pink yeast group. No clear clustering, with more than 50 % band similarity was found (Fig. 3 A). One cluster was found among the beige and orange isolates, with 6 and 12 isolates respectively in each cluster (Fig. 3 B and C), and two clusters, with 5 and 6 isolates, were found in the white yeast group (Fig. 3 D). Isolates from different collection dates were not evenly distributed among these clusters. The first cluster includes isolates that were collected on two grape cultivars from the 7th (704, 724 and 719) and the 8th harvest dates (802, 815). The second cluster includes yeasts from the three cultivars that were harvested on the second (229,201 and 221), 4th (424 and 425) and 5th (514) date. Only one cluster (in the beige group) contained isolates that were collected on 5 different dates from the three cultivars.

RAPD analysis of yeasts isolated in 1999 was done with two primers (Op-C5 and Op-C20) to define the variability among isolates that were collected from single berries. Yeast colonies developed in 50-100 % of the Petri dishes plated with wash water from individual berries. Morphologically similar pairs of isolates originating from the same berry were compared using RAPD. In 64, 63 and 47 % of the isolate pairs from Cabernet Sauvignon, Muscat and Colombard berries, respectively, identical electrophoretic patterns were found (Tab. 1).

Yeast identification: Representatives of the white yeast group, collected in 1997, with less then 50 % band similarity were sent to Holland for identification (Tab. 2). All except two isolates were identified as different species.

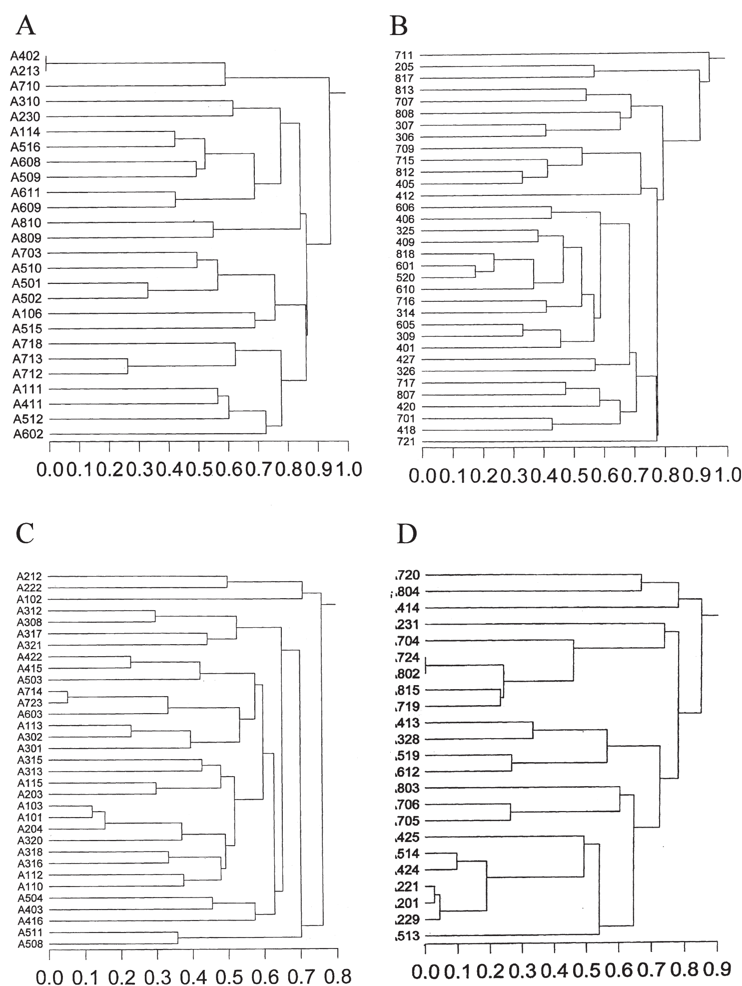

Fig. 3: Dendrogams of similarity (of isolates collected in 1997) generated from matrix of similarity coefficients using Nei's resembling function. Data are based on presence or absence of common electrophoretic fragments obtained with two RAPD and two ap-PCR primers. **A**: pink yeasts, **B**: beige, **C**: orange and **D**: white yeasts. - The first digit in the isolate identification number represents the nth sampling. RAPD primers Op-C5 and Op-D20, ap-primers - $(GACAC)_3$ and $(CAG)_5$.

Table 1

Isolate diversity on single berries sampled from three grape cultivars during 1999

	June		July		August		September		Avg.[3]
	Berries[1]	Identicals[2]	berries[1]	Identicals[2]	berries[1]	Identicals[2]	berries[1]	Identicals[2]	(%)
Cabernet Sauvignon	10	9	4	1	5	2	4	4	64
Muscat	7	6	2	1	4	2	3	2	63
Colombard	5	4	3	1	10	4	3	1	47

[1] The number of berries, collected on each sampling date from which morphologically identical pairs of yeasts were sampled.
[2] The number of pairs that were found to have identical electrophoretic patterns using RAPD-PCR with two primers (OpC5 & OpD20).
[3] The average percentage of cases, over the four sampling dates for each cultivar, in which identical isolates were found on the berries.

Discussion

In this work we have shown the great qualitative and quantitative variability among yeast strains native to grape berry surfaces. Screening of yeast isolates with RAPD PCR and ap-PCR proved to be quick, simple and efficient and identical isolates were easily recognized. By creating a dendrogram based on the percentage of common RAPD bands, different isolates were selected and then identified by traditional methods. Isolates that shared <50 % of the bands in their RAPD-PCR pattern were identified as different species with only one exception. Following molecular

Table 2

Identification of selected white yeast isolates (collected in 1997) by the "Centraalbureau voor Schimmelcultures" (Baarn, The Netherlands)

Isolate	Grape cultivar	Collection Date (97)	Identification
413	Cabernet[1]	17/7	*Citeromyces matritensis* (Santa Maria) Santa Maria
414	Cabernet[1]	17/7	*Debaryomyces hansenii* (Zopf) Lodder & Kreger-Rij var. Fabryii (Ota) Nakase & Suzuki
513	Cabernet[1]	31/7	*Candida pulcherima* (Lindener) Windisch
519	Cabernet[1]	31/7	*Candida guilliermondii* (Castellani) Langeron & Guerra
706	Cabernet[1]	26/8	*Pichia klyveri* Bedford ex Kudryavtsev
803	Cabernet[1]	9/9	*Issatchenkia terricola* (van der Walt) Kurtzman *et al.*
804	Cabernet[1]	9/9	*Hanseniaspora guilliermondii* Pijper
816	Colombard	9/9	*Hanseniaspora uvarum* (Niehaus) Shehata*et et al.*
425	Colmbard	17/7	*Citeromyces matritensis* (Santa Maria) Santa Maria
720	Colmbard	26/8	*Kloeckera apis* M.T. Smith *et al.*
229	Muscat[2]	16/6	*Candida famata* (Harrison) Meyer & Yarrow var. flareiri
231	Muscat[2]	16/6	*Metschnikowia reukauffii* Pitt & Miller

[1] Cabernet Sauvignon
[2] Muscat of Alexandria

screening, 11 different species were identified by traditional methods among the white yeast group alone, more than the total number reported by either Davenport (1974) or Rosini (1982) (8 and 10 species, respectively). Most of these isolates (e.g. *Debaryomyces hansenii, Candida* spp., *Kloeckera* spp. and *Hanseniaspora* spp.) have been reported as frequent residents of grape berries in most regions where vineyard microflora was studied, but one (*Citeromyces matritensis*) belongs to genera that have not yet been mentioned with regard to grape microflora (Pretorius 1999). This suggests that molecular tools are efficient in tracking even rare strains. The rather diverse situation is different from that found by Droby *et al.* (1999) on grapefruit surfaces, where two major groups of amplification patterns predominated. This is probably because grape surfaces are a very rich food source containing various sugars, organic and amino acids as well as vitamins. Padget and Morrison (1990) have shown the effect of grape berry exudates on the development of *Botrytis cinerea* mycelium. It is reasonable that many different yeast types may find their energy source on the berry surface and proliferate there.

Comparisons drawn between the three grape cultivars indicate that yeast populations tended to be lower on Colombard berries than on Cabernet Sauvignon or Muscat of Alexandria berries (statistically significant for three of the sampling dates). The biological significance of greater numbers of CFU could be that cells adhere to some grape cultivars better than to others or that cells that accidentally land on the berry surface encounter a more favorable niche for budding on certain cultivars. We tried to clarify this by sampling isolates from single berries and using the molecular screening method described herein. The idea was to see if there were fewer cases with repetitive electrophoretic patterns among the isolates collected from the grape cultivar that supports a smaller yeast flora (Colombard). Our results suggest that this might indeed be the case, as on 64 and 63 % of the berries of Cabernet Sauvignon and Muscat of Alexandria respectively the isolates we collected appeared in more than one copy as compared to only 47 % of the Colombard berries. One of the objectives of this work was to find native strains that can proliferate on the berry surface in the changing vineyard environment. Following the work of Droby *et al.* (1999), we assumed that yeast types that are better adapted to grape surfaces would be found abundantly and therefore probably be superior biological control agents. Most of the strains that were isolated from grapefruit surfaces belonged to 6 yeast species and some of them shared identical electrophoretic patterns. In the present study, in spite of the effort to select strains from morphologically identical colonies, isolates with identical electrophoretic patterns occurred very rarely on the same bunch and only on about half of the sampled berries. The large number of species identified after the screening process can explain why identical strains were rare and suggests that in order to identify a dominant strain a much greater number of isolates needs to be screened.

Our sampling period was from fruit set to harvest. Clusters of strains in the dendrogram tended to be correlated more with the date of sampling then with the grape cultivar. Most of the yeast clusters in the dendrograms include isolates that were collected from the three grape cultivars on two or three sampling dates. One of the yeast clusters includes only isolates from the last two sampling dates and two clusters include only isolates from the early developmental stages. The accumulation of sugar after veraison, which started between the fourth and fifth sampling dates (in all three cultivars) might have contributed to the clustering pattern of the isolates collected over the season.

The results of this study prove that the methods used can help in screening a large number of isolates in a relatively short period, making sure none are lost and no two identical copies are being held, thus facilitating ecological studies or search for isolates with specific traits.

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