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Participation of Vineyard Soil Yeasts in the Spontaneous Must Fermentation †

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Abstract: The main objective of this work was to gather evidence as to whether the yeasts present in ripe grapes before harvest and those found in spontaneous wine fermentations came from the vineyard soil which could then be regarded as a natural reservoir for these yeasts. Two types of management system were tested in each vineyard: conventional tillage (CT), and no-tillage with natural green cover vegetation (NV), both under semi-arid rainfed conditions. Bacteria isolated from the grapes all corresponded to three genera that were very abundant in soil samples taken just before grape harvest. The amounts of fermentative yeasts in vineyard soil increased significantly during the dates close to harvest. Some yeasts were isolated from soils and spontaneous fermentations (*Saccharomyces* and *Lachancea*), while others were only isolated from fermentations (Apiculated yeasts as *Hanseniaspora*) or from soils (*Torulaspora*). *Saccharomyces* yeasts were isolated from vineyard soil only after grape harvest. The analysis of sterile-must fermentations inoculated with soil samples showed that soil was not the origin of the most abundant fermentative yeasts in spontaneous grape fermentations (*Saccharomyces* and *Hanseniaspora*). In contrast, other fermentative wine yeasts such as *Lachancea* and *Torulaspora* seemed to be permanently resident in the vineyard soil, especially in the NV vineyard. The yeasts involved in spontaneous grape fermentation (mainly *Hanseniaspora* and *Saccharomyces*) must have reached ripe grapes by a process other than the mere accumulation of wind-borne soil dust. Conversely, vineyard soil did appear to be a permanent natural reservoir for *Torulaspora* and *Lachancea* yeasts, especially in the NV-vineyard.

Keywords: vineyard soil; soil management; natural reservoir; wine yeasts; *Saccharomyces*; *Lachancea*; *Torulaspora*; spontaneous grape fermentation

1. Introduction

The vineyard soil could be a natural reservoir for microorganisms involved in spontaneous wine fermentations. There should occur a reciprocal flow of microorganisms between the soil and the grape for it. The management system used in the vineyard could influence this flow. In particular, the management regime could determine the evolution of spontaneous fermentation of the must, especially at the beginning of the grape harvest season before fermentative yeasts have become ubiquitous in wineries and vineyards. It has been found that total number of culturable microorganisms in a vineyard soil suffered seasonal fluctuations related to weather and the phenological state of the vines. Moreover, the amount of most microorganisms was mainly affected by the soil management regime. However, only the yeast population was mainly dependent on the

phenological state of vines, with its amount seeming to be related to the availability of fermentable sugars from ripe grapes and to human activity during grape harvest [1]. *Saccharomyces cerevisiae* and other yeasts such as *Torulaspora* seem not to be airborne, thus requiring a carrier to spread. Also, it is still unclear which are the safe reservoirs where yeasts can overwinter and stay alive when no grapes or musts are available, and whence they can be taken by carrier vectors to spread in the vineyard. A possible natural reservoir of yeasts common to all wine regions around the world may be the vineyard soil, and wind-blown soil dust would then be an ubiquitous vector for their dissemination. An input of soil bacteria to grapes has also been proposed [2], suggesting that the relationship between soil microorganisms and wine terroir should be further examined. Fermentative yeast growth in soil is possible, however, close to the harvest season because of the presence of sugars from damaged grapes [3].

As is the case with *S. cerevisiae*, the yeast *Torulaspora delbrueckii* is also associated with several human food processing activities, such as winemaking. In terms of biotechnological advantages, *T. delbrueckii* is the second of all the fermentative yeast species most likely to be chosen for winemaking after *S. cerevisiae*. Unfortunately, *T. delbrueckii* has less growth rate and fermentation vigour than *S. cerevisiae* in grape must. Nevertheless, full domination and completion of wine fermentation has been found only for some *T. delbrueckii* killer strains and its inoculation has some positive effects on wine as increasing such interesting compounds as lactones [4–6].

The aims of this work were to better understand the reciprocal flow of microorganisms between vineyard soil and ripe grapes, to investigate the possible effects of vineyard management on spontaneous wine fermentation, and to determine whether the vineyard soil is a reservoir for wine fermentative yeasts (mainly *S. cerevisiae* and *T. delbrueckii*) found in spontaneous must fermentation at the beginning of each harvest season [7].

2. Materials and Methods

Two vineyards with different management systems: conventional tillage (CT) and no-tillage with natural green cover vegetation (NV) were analysed. To compare the two types of soil management systems, a randomized design was used as described previously [1]. Soils were sampled three months before harvest (3mBH), one week before harvest (BH), one week after harvest (AH), and three months after harvest (3mAH). Two sub-samples were randomly collected under the vines (UV) and in the inter-row area (IR).

For detection of culturable soil microorganisms, soil samples were passed through a 2-mm sterile sieve, diluted with sterile distilled water, and spread onto different agar culture media (YEPD, TSA, SC, AZO, potato-glucose with 10% tartaric acid, rose-bengal with chloramphenicol, and glucose-Saboraud with chloramphenicol) as described previously [1]. For molecular analysis, DNA was extracted from bacteria and cultivable yeasts from soils and grapes, and the corresponding 16S rRNA and 18S rRNA genes were amplified (PCR) respectively, according to the methods specified by [7], to their subsequent sequencing and identification. Procedure for Mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP) analysis and determination of yeast killer activity were performanced that described previously [7].

For spontaneous grape fermentations, undamaged grapes were selected from each vineyard. Must density and °Brix were monitored every day. Samples were taken from each fermentation – the beginning (BF), tumultuous stage (TS), and end (EF) of fermentation – for yeast isolation on YEPD plates. For fermentations of sterile grape must inoculated with soil samples, sterile must was inoculated with soil samples (3 mg/mL) and incubated at 20 °C. These fermentations were monitored and sampled as described above. For confirmation of yeast type assignment, the mtDNA-RFLP was analysed for yeast isolates from each type of fermentation.

Analysis of variance (ANOVA) and the Duncan test (at $p < 0.05$) were used to detect significant differences. Software package SPSS version 19.0 for Windows (Chicago, IL) was used.

3. Results and Discussion

3.1. Presence of Culturable Yeasts in Vineyard Soils and Grapes

The apiculate yeasts Hanseniaspora were the most frequent in grape must samples, however, were not detected in the soil samples. Saccharomyces yeasts appeared in must and soils samples, but only in those taken after harvest. As in the case of Saccharomyces, Lachancea yeasts were also found in both samples, but this latter species was found in soil samples before and after harvest. Torulaspora yeasts were only isolated from soil samples (Table 1). At most locations and dates, the quantity of non-Saccharomyces yeasts was greater in the NV- than in the CT-vineyard soil, but it was similar for Saccharomyces yeasts in all cases. Nonetheless, the quantity of non-Saccharomyces increased before and after harvest in both vineyards, while Saccharomyces yeasts only increased after grape harvest (not shown). NV management is a conservation agriculture practice that generally increases soil organic matter [8], and it may therefore also increase soil microbial community and diversity. Soil's yeast populations seem to depend mainly on the phenological state of the vine and human activity when harvesting grapes, being related to availability of fermentable sugars from ripe grapes [1]. In the working conditions of this study, the amount of Torulaspora and Lachancea were influenced by soil management, while that of the Saccharomyces and Hanseniaspora yeasts were not, indicating that the two former are living in vineyard soil, but the most abundant wine fermentative yeasts probably come from somewhere else.

Table 1. Average percentages of mainly culturable yeasts isolated from soil and grape must samples.

Species	Soil	Grape must
Hanseniaspora uvarum	nd*	48.75
Lachancea waltii	4.4	11.25
Saccharomyces cerevisiae	6.3	19
Torulaspora delbrueckii	30.6	nd
Others identified	54.3	21
Others unidentified	4.4	nd

**nd, non-detected.*

3.2. Culturable Microorganisms Present in Spontaneous Fermentation of Crushed Grapes and Sterile Must Inoculated with Soil Samples

Non-Saccharomyces were the dominant yeasts throughout all spontaneous grape fermentations, although most of them decreased as the Saccharomyces yeast population grew. The exception was the apiculated yeasts (Hanseniaspora ssp) that increased during fermentation similarly to Saccharomyces. Moulds and bacteria also appeared at the beginning of fermentations (<2 × 10^4 CFU/mL) to disappear later (Figure 1). All bacteria isolated from freshly must presented at the beginning of spontaneous fermentation, belonged to three very abundant genera in vineyard soils: Pseudomonas, Stenotrophomonas, and Bacillus. These three genera were always isolated from soil samples taken before grape harvest (not shown).

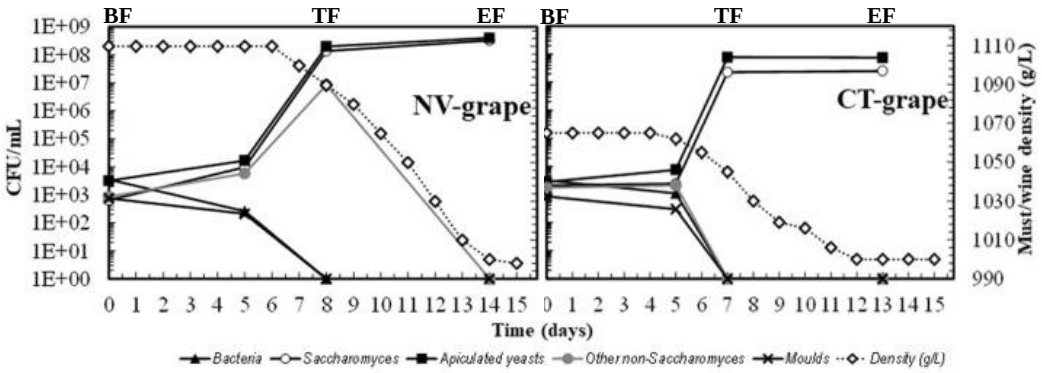

Figure 1. Fermentation kinetics and monitoring of viable microorganism during spontaneous fermentation of grapes from two vineyards: natural vegetation (NV) and conventional tillage (CT). Samples: BF, beginning of fermentation; TF, tumultuous fermentation; EF, end fermentation.

The finding that all bacteria isolated from crushed grapes belonged to some of the most abundant genera found in vineyard soils indicates a possible contamination of grapes with edaphic microorganisms, probably by deposition of dust from vineyard soil. However, it seems that most yeasts found in vineyard soil are either not airborne or do not adhere strongly enough to the grape surface to remain there for long. These circumstances would explain why most yeasts that were abundant in soil samples were not found in must (Table 1 and Figure 1). Beside *Saccharomyces*, several non-*Saccharomyces* yeasts were found during all stages of spontaneous fermentations, as described elsewhere [9]. However, most fermentative yeasts found in grape fermentations were not found in soil samples. This may be because they could adhere to the grape surface probably after being transported to the surface of ripe grapes by visiting birds or insects.

On the other hand, all fermentations inoculated with soil samples from NV-vineyard taken under-vine area (UV) were quick and complete in all sampling date. In contrast, most fermentations inoculated with soil samples from the CT-vineyard (under-vine and inter-row area) or from the NV inter-row area were slow and incomplete. The exceptions among were fermentations inoculated with soil samples collected after grape harvest (AH), of which most were quick, and all were complete (Table 2). The completed fermentations contained mainly *Torulaspora* or, in those fermentations inoculated with soil samples taken after grape harvest, *Torulaspora* plus *Saccharomyces* yeasts. The exceptions in these latter cases were fermentations inoculated with samples taken after grape harvest from the NV-IR location which mainly contained *Saccharomyces* plus some *Torulaspora* and *Lachancea* (Figure 2), and were the slowest fermentations of all those that were completed (Table 2). This may reflect some negative interaction between different yeast populations, in fact these two last yeasts showed killer phenotype against *Saccharomyces* in the MB-agar plates test (Figure 3). This killer activity may have stopped *Saccharomyces* dominating when these non-*Saccharomyces* yeasts were most abundant in vineyard soil. Non apiculated yeasts were detected in any soil-inoculated fermentation.

Fermentation trials of sterile must inoculated with soil samples indicated that efficient fermentative yeasts (including *S. cerevisiae* and *T. delbrueckii*) were present in all vineyard locations after grape harvest, probably because of the availability of sugars and the input of some yeast species (such as *S. cerevisiae*) from ripe grapes that had fallen to the ground. They then disappeared during the winter-spring seasons either because of tillage in the CT-vineyard soil, or because of the lack of sufficient quantities of sugars and absence of soil protection by the vine canopy in the NV-IR soil. Exceptions were the NV-UV location for *T. delbrueckii* and the rest of the locations for *L. waltii*, where these yeasts appeared to remain throughout the year (Figure 2). This could be because of the absence of tillage and protection of the soil by natural vegetation and vine canopy.

All these results indicate that soil dust is not the principal origin of the major spontaneous wine fermentation yeasts (*Hanseniaspora* and *Saccharomyces*), at least under our working conditions and probably in any other situation that avoids any major contamination of the grape harvest with soil. Except for *Lachancea*, our results thus clearly indicate that soil was not the principal source of fermentative yeasts found in our spontaneous grape fermentations and came from somewhere else. Possible vectors might have been birds or insects that had previously visited other fruits or their fermenting juice, or insects that harbour these yeasts in their gut.

Table 2. Mean T15 and T100 values (days) of must fermentations inoculated with soil samples.

Soil Management - Location	3MBH		BH		AH		3MAH	
	T15	**T100**	**T15**	**T100**	**T15**	**T100**	**T15**	**T100**
NV-UV	4.85 a	14.0 a	4.50 a	15.0 a	5.26 a	15.5 a	6.17 a	17.6 a
NV-IR	13.2 b	35.0 b	7.50 b	35.0 b	6.93 b	19.6 b	11.1 b	35.0 b
CT-UV	11.5 b	35.0 b	7.70 b	35.0 b	5.13 a	13.9 a	10.2 b	35.0 b
CT-IR	12.8 b	35.0 b	7.90 b	35.0 b	5.93 a	14.6 a	11.1 b	35.0 b

Soil management: NV, no-tillage with natural vegetation; CT, conventional tillage. Location in the vineyard: UV, under-vine area; IR, vine inter-row area. Sampling date: 3mBH, three months before grape harvest; BH, one week before harvest; AH, one week after harvest; 3mAH, three months after harvest. T15, time needed to ferment 15% of the total sugars present in the must; T100, time needed to ferment 100% of the total sugars or to reach a non-fluctuating level. The data are the mean values of three independent experiments. The standard deviations were less than 14% of the corresponding mean. Values with the same letter within a column are not significantly different at the $p < 0.05$ level of probability.

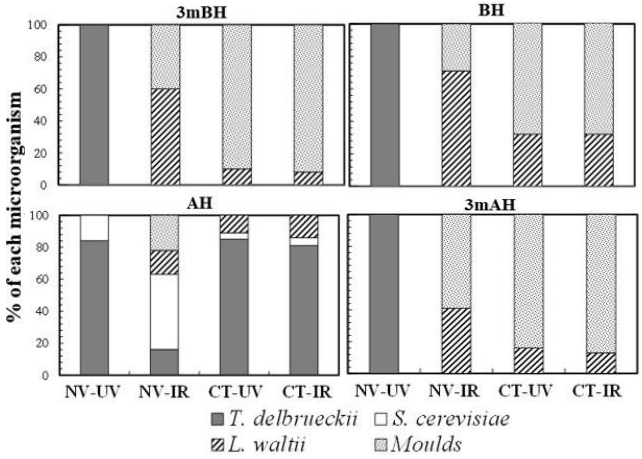

Figure 2. Frequencies of culturable moulds and yeasts isolated during a set of fermentation trials of sterile must inoculated with soil samples from NV- and CT-vineyards collected at different locations (UV, under-vine area; IR, vine inter-row area) at different times (3mBH, three months before grape harvest; BH, one week before harvest; AH, one week after harvest; 3mAH, three months after harvest).

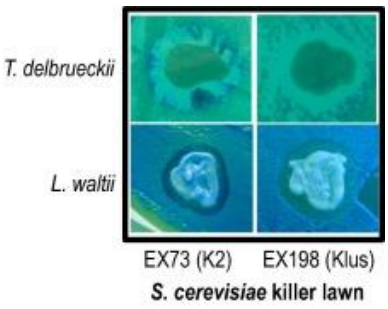

Figure 3. Killer phenotype assay of *T. delbrueckii* and *L. waltii* killer yeasts isolated from vineyard soil against *S. cerevisiae* strains. The assay was done on MB agar plates seeded with standard *S. cerevisiae* killer K2 (EX73) and Klus (EX198) strains [10]. The assay conditions were pH 4 and 20 °C.

4. Conclusions

The yeasts (mainly *Hanseniaspora* and *Saccharomyces*) involved in spontaneous grape fermentation must have reached ripe grapes by a process other than the mere accumulation of wind-borne soil dust. Conversely, vineyard soil did appear to be a permanent natural reservoir for *Torulaspora* and *Lachancea* yeasts, especially in the NV-vineyard. Since *Torulaspora* does not seem to be airborne or capable of adhering strongly enough to grape surface in our working conditions, its

contingent presence in spontaneous wine fermentation may be regarded as indicative of a similarly contingent contamination with vineyard soil. The present results can be of help in designing effective strategies to isolate and select new wine yeast strains from vineyards, especially unconventional winemaking yeasts such as *Torulaspora* and *Lachancea*.

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Conflicts of Interest: The authors declare no conflict of interest.

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