Sour rot-damaged grapes are sources of wine spoilage yeasts

André Barata[1], Sara González[2], Manuel Malfeito-Ferreira[1], Amparo Querol[2] & Virgílio Loureiro[1]

[1]Laboratório de Microbiologia, Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, Technical University of Lisbon, Lisboa, Portugal; and [2]Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, Valencia, Spain

Abstract

Yeast species of sound and sour rot-damaged grapes were analysed during fermentation and grape ripening in the vineyard, using general and selective culture media. During 2003 and 2004 vintages, microvinifications were carried out with sound grapes to which different amounts of grapes with sour rot were added. The wine spoilage species *Zygosaccharomyces bailii* was only recovered during fermentations with sour rot, reaching $5.00 \log \mathrm{CFU\, mL}^{-1}$ (2003) and $2.48 \log \mathrm{CFU\, mL}^{-1}$ (2004) at the end of fermentation. The study of yeast populations during the sour rot ripening process (2005 vintage) showed that the veraison-damaged grapes always exhibited higher total yeast counts and a much greater diversity of species. From a total of 22 ascomycetous species, 17 were present only in damaged grapes. The most frequent species were *Issatchenkia occidentalis* and *Zygoascus hellenicus*. The spoilage species *Z. bailii* and *Zygosaccharomyces bisporus* were consistently isolated exclusively from damaged grapes. This work demonstrates that one of the most dangerous wine spoilage species, *Z. bailii*, is strongly associated with sour rot grapes and survives during fermentation with *Saccharomyces cerevisiae*. The use of selective media provides a more accurate characterization of grape contamination species.

Introduction

The effect of non-*Saccharomyces* yeasts on fermentation and wine quality is one of the principal issues currently studied in countries with an oenological tradition (Jolly *et al.*, 2003). Many ecological studies in different wine regions of the world have identified the major yeast species that develop during fermentation (Povhe-Jemec *et al.*, 2001; Jolly *et al.*, 2003). However, the study of the microbial succession during grape juice fermentations is usually applied when sound grapes are the raw material for wine production (Fleet, 2003).

The yeasts associated with wine may be classified in empirical groups according to their technological properties (Loureiro & Malfeito-Ferreira, 2003). Oxidative basidiomycetous yeasts, without any oenological interest, such as *Sporobolomyces*, *Cryptococcus*, *Rhodotorula*, *Filobasidium* spp. and *Aureobasidium pullulans*, are mostly prevalent in the vineyard environment (soil, bark, leaves, grapes) (Davenport, 1974; Sabate *et al.*, 2002; Subden *et al.*, 2003; Prakitchaiwattana *et al.*, 2004; Renouf *et al.*, 2005). Among ascomycetes, apiculate weakly fermentative yeasts (*Hanseniaspora* and

Kloeckera spp.) and oxidative yeasts (mostly *Candida*, *Pichia* and *Metschnikowia* spp.) are predominant on ripe sound grapes (Davenport, 1974; Sabate *et al.*, 2002; Jolly *et al.*, 2003; Subden *et al.*, 2003; Prakitchaiwattana *et al.*, 2004; Renouf *et al.*, 2005). The last group includes fermentative species, which are rare contaminants of grapes but represent the most dangerous wine spoilers, such as *Saccharomyces cerevisiae*, *Torulaspora* spp., *Zygosaccharomyces* spp. and *Dekkera* spp. (Fleet *et al.*, 2002).

Significant growth of non-*Saccharomyces* yeasts in fermentations has been associated with off-character production in wines and/or stuck and sluggish fermentations (Bisson, 1999). In addition, there is little work on the survival of yeast species dangerous to bottled wine stability during fermentation. In fact, wine spoilage yeasts such as *Dekkera/Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* are usually studied for their activities during storage and after bottling stages (Loureiro & Malfeito-Ferreira, 2003).

The occurrence of fermentative species, such as *S. cerevisiae*, is higher when grape skin is damaged and juice escapes onto the grape surface (Mortimer & Polsinelli, 1999). Bearing in mind that wine spoilage species are also

fermentative, Loureiro & Malfeito-Ferreira (2003) hypothesized that the knowledge of their dissemination could greatly improve if more attention was given to the microbiology of damaged grapes. The microbial ecology of damaged grapes is poorly studied and it is not clear whether damaged grapes are significant vehicles of dangerous spoilage microorganisms (Loureiro & Malfeito-Ferreira, 2003). Damaged grapes include those attacked by several types of rot, insects, hail and/or heavy rains. In particular, sour rot is a disease characterized by grape pulp browning, disaggregation of the internal tissues, detachment of the rotten berry from the pedicel, grape dropping and a strong smell of ethyl acetate (Bisiach *et al.*, 1982; Guerzoni & Marchetti, 1987). It may affect a large proportion of the crop and is frequently the main cause for grape damage. The most frequently isolated yeast species from sour rot-damaged grapes are *Hanseniaspora uvarum*, *Candida stellata*, *Metshnikowia pulcherrima*, *Candida krusei* and *Kloeckera apiculata* (Bisiach *et al.*, 1986; Guerzoni & Marchetti, 1987).

Until now, studies of the microbial communities of sound and damaged grapes were carried out using general-purpose culture media leading to the underestimation of wine-spoiling species. Spoilage species usually grow more slowly in general-purpose culture media and hence tend to be underestimated in samples heavily contaminated by other microorganisms (Loureiro *et al.*, 2004). Barata *et al.* (2008) showed that the use of selective media was crucial to characterize the yeast ecology of honeydew excreted by mealy bugs on grape surfaces and preliminary results demonstrated the occasional presence of *Z. bailii* in sour rot-damaged grapes.

The purpose of this work was to evaluate the occurrence of the wine spoilage species *Z. bailii* in sour rotten grapes and to check its survival during grape must fermentation.

Material and methods

Sample collection and wine fermentations

During the 2003 and 2004 vintages, healthy and sour rot-affected bunches (Trincadeira Preta red grape variety) were collected at the time of harvest from an experimental vineyard of the Instituto Superior de Agronomia (Lisbon, Portugal). Grapes were transported to the laboratory and stored in sterile bags at 5 °C. Three sets of microvinifications (red style fermentation with grape skin contact) were carried out in 2-L Schott flasks at 25 °C using sound grapes to which different amounts of sour rot-damaged grapes were added: (1) 100% (p/p) of sound grapes (control); (2) 10% (p/p) of damaged grapes; and (3) 30% (p/p) of damaged grapes. After addition of 80 mg L^{-1} potassium metabisulphite to emulate winery conditions, grape musts were inoculated with 10^6 cells mL^{-1} of a commercial *S. cerevisiae* (Fermivin®,

DSM, Delft, the Netherlands), and fermentations were carried out until °Brix determinations indicated the end of fermentation.

Yeast isolation during fermentations

Samples from the fermenting musts were taken during the beginning, the middle and the end of fermentations, and yeasts were recovered after decimal dilutions in peptone water (Merck, Darmstadt, Germany). The criteria to define the middle and end of fermentation were based on sugar consumption. The isolation and enumeration of yeasts was carried out by spreading onto plates of several culture media: GYP (20 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 20 g L^{-1} agar) and Mann Rogosa Sharpe (52 g L^{-1} MRS, Oxoid) general-purpose media in order to isolate *Saccharomyces* and non-*Saccharomyces* species and *Dekkera-Brettanomyces* differential medium (DBDM) (Rodrigues *et al.*, 2001) and *Zygosaccharomyces* differential medium (ZDM) (Schuller *et al.*, 2000) selective/differential media for the isolation of *Dekkera/Brettanomyces* and *Zygosaccharomyces* species, respectively. To avoid growth of bacteria, chloramphenicol (Sigma) was added to the media at 100 mg L^{-1}. The plates were incubated at 25 °C for 7 (GYP and MRS) and 20 days (DBDM and ZDM). Plates containing between 30 and 300 yeast colonies were examined, and all colony morphological types were counted, isolated and purified by streaking on GYP plates.

The yeast isolates were preserved on YM agar slants (3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 5 g L^{-1} peptone, 10 g L^{-1} glucose, 20 g L^{-1} agar) until later analysis, stored at 4 °C and subcultured every 3 months.

Analysis of natural yeast community from sound and sour rotten grapes

To quantify the yeast community of healthy and sour rot-affected grapes, 100-g samples of sound and damaged berries were aseptically removed from single sound and sour rot-affected bunches during the 2004 vintage. The berries were placed in a sterile stomacher bag and crushed in a stomacher blender (IUL, Barcelona, Spain). The grape musts were subsequently diluted in peptone water and the natural yeast community was analysed by plating (duplicate) onto GYP, DBDM and ZDM plates, as described previously.

Yeast community analysis during the sour rot ripening process

In the 2005 vintage, two sets of three vines from the same red grape variety and experimental vineyard were selected for taking samples of sound or sour rot-affected bunches at three different ripening phases: (1) after veraison; (2)

middle of ripening; and (3) before the harvest. A total of three bunches from each vine set were collected aseptically and transported to the laboratory. Individual grape berries were randomly and aseptically removed from the bunches and combined to give 300-g samples. The berries were crushed in a stomacher blender, and grape musts were directly diluted in 10-fold series in peptone water. The yeasts recovered from grape samples were analysed by spreading (duplicates) 0.1 mL of the appropriate dilutions onto GYP, DBDM and ZDM plates. Colonies were counted after incubation at 25 °C for 7 (GYP) and 20 days (DBDM and ZDM), and yeast isolation was performed as described previously.

Biochemical characterization and identification

The yeast isolates during the 3-year study were characterized previously according to the urease reaction. Only the urease-negative strains (ascomycetes) were identified by PCR amplification of the 5.8S-internal transcribed spacer (ITS) rRNA gene region and subsequent restriction analysis (Esteve-Zarzoso *et al.*, 1999). This region was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Yeast cells were picked from a fresh colony (48-h-old) and resuspended directly in the PCR mixture. PCR reactions were performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) thermocycler as follows: a first step at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min, with a final extension cycle at 72 °C for 10 min. Two units of DNA polymerase (Biotools, Madrid, Spain) were added to each reaction. For restriction reactions of the 5.8S-ITS region, 10 μL or 0.5–10 μg of amplified PCR product was incubated for 5 h at 37 °C with 10 U of CfoI, HaeIII and HinfI (Roche Molecular Biochemicals, Mannheim, Germany) restriction endonucleases. PCR products and their corresponding restriction fragments were separated on 1.4% and 3% agarose gels, respectively, detected by ethidium bromide staining and photographed under UV light with a GelDoc system (Bio-Rad, CA). Fragment sizes were estimated by comparing their mobility against a 100 bp DNA ladder (Bioron, GmbH, Ludwigshafen, Germany).

The strains of the *Lachancea* clade (*Zygosaccharomyces fermentati*, *Zygosaccharomyces cidri*, *Kluyveromyces thermotolerans* and *Kluyveromyces waltii*) with similar restriction profiles and the yeast isolates with misidentified restriction profiles were identified by sequencing of the D1/D2 variable domains of the large subunit rRNA gene. The D1 and D2 domains of the 26S rRNA gene were amplified using the external primers NL-1 (5′-GCATATCAATAAGCGGAGGA AAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell, 1993). PCR reactions were performed as follows: a first denaturation step at 95 °C for 5 min, followed by

40 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 30 s, with a final extension of 10 min at 72 °C. The PCR products were purified using a GFX PCR and a Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions and then sequenced directly. Primers NL-1 and NL-4 were used in the sequencing reactions to read both DNA strands of D1 and D2 domains of the 26S rRNA gene. Sequences of the D1/D2 26S rRNA gene were edited and assembled using BIOEDIT SEQUENCE ALIGNMENT EDITOR version 7.0.1 software (Hall, 1999), and then subjected to a GenBank BLASTN search to retrieve sequences of closely related taxa. Distinction of species with identical restriction profiles or similar 26S rRNA gene sequences was carried out using biochemical reactions (Kurtzman & Fell, 1998) and the DBDM medium. *Hanseniaspora uvarum* was differentiated from *Hanseniaspora guilliermondii* and *Dekkera anomala* by growth on GYP agar plates incubated at 37 °C and growth on DBDM plates. *Zygosccharomyces fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii* were differentiated by growth on GYP agar plates incubated at 37 and 40 °C, and growth with D-galactose and 0.01% cycloheximide.

Results

Identification of yeast isolates

The isolates recovered from grape and must samples were first tested with the urease reaction to estimate the proportion of ascomycetes (urease negative) and basidiomycetes (urease positive). Because basidiomycetous yeasts are not regarded as relevant to winemaking (Loureiro & Malfeito-Ferreira, 2003), no further identification analysis was done for these strains.

A total of 426 yeast strains were isolated from grape or grape must samples in 2003, 2004 and 2005 vintages. A total of 188 and 125 yeast strains were isolated from Trincadeira Preta sound and sour rot-contaminated grape must fermentations during 2003 and 2004 vintages, respectively. In the 2005 vintage, 113 yeast isolates were recovered from samples of sound or sour rot-affected bunches in three different ripening phases. The phenotypic urease activity test allowed the distinction of 390 ascomycetes yeast strains, which were analysed by performing a PCR-restriction fragment length polymorhism (RFLP) analysis of the 5.8S-ITS rRNA gene region (restriction fragments not shown). Using CfoI, HaeIII and HinfI restriction endonucleases, 23 different restriction patterns were generated, from which it was possible to assign 18 species according to the restriction patterns in the Yeast-id database (http://www.Yeast-id.com). These corresponded to *Candida amapae*, *Candida diversa*, *Candida ethanolica*, *C. stellata/zemplinina*, *Candida tropicalis*, *Candida vanderwaltii*, *Hanseniaspora osmophila*, *Issatchenkia*

occidentalis, *Issatchenkia orientalis*, *Issatchenkia terricola*, *Pichia galeiformis*, *Pichia guilliermondii*, *Pichia kluyveri*, *Pichia membranifaciens*, *S. cerevisiae*, *Zygoascus hellenicus*, *Z. bailii* and *Zygosaccharomyces bisporus*. However, this method could not identify species when the restriction bands differed by < 50 bp, necessitating further characterization by sequencing or biochemical tests. One group included *H. guilliermondii*, *H. uvarum* and *D. anomala*. Their distinction was made based on the observed growth on agar plates incubated at 37 °C and by the absence of growth on plates of DBDM, indicating the presence of *H. guilliermondii*. Absence of growth on DBDM plates and agar plates at 37 °C indicated the presence of *H. uvarum* or its anamorph *K. apiculata*. Another group of similar restriction profiles included the former species *Z. fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii*. The recent genus *Lachancea* includes the above-mentioned four species with similar restriction profiles. All the isolates with these similar restriction profiles displayed positive growth with D-galactose and at 37 °C, and did not grow under 40 °C and 0.01% cycloheximide. These physiological results are consistent with the old species *K. thermotolerans* and not with *Z. cidri*, *Z. fermentati* or *K. waltii*. Moreover, their 26S rRNA gene sequence results (EU541357 GenBank accession number) yielded 99% similarity to *Lachancea* sp. CBS 6924 (EF463105) as the closest relatives. Therefore, we assigned those strains to *Lachancea thermotolerans*.

Because of the absence of species assignment of their restriction patterns in the Yeast-id database, *Candida oleophila*

(EU541359) and *Hanseniaspora occidentalis* (EU541358) were identified by sequence analysis of the 26S rRNA gene D1/D2 domain, yielding 100% similarity to *C. oleophila* CBS 2219 (AY528671), and 99% to *H. occidentalis* var. *citrica* CBS 6783 (AJ973092), respectively.

Yeast community dynamics during alcoholic fermentation

Yeast species populations recovered from three sets of microvinifications with different proportions of sour rot-damaged grapes at the beginning, the middle and the end of fermentation are presented in Table 1. The most frequent species was *S. cerevisiae* as expected because of starter addition. However, the identification method by RFLP analysis is not adequate for intraspecific typing; hence, we cannot guarantee that all identified strains belong to the *S. cerevisiae* starter. In the control set (100% sound grapes), besides *S. cerevisiae*, only basidiomycetous yeasts, *H. uvarum* and *C. diversa* strains were isolated at the initial stages of fermentation during the 2004 vintage, while in the 2003 vintage only *S. cerevisiae* was isolated.

The alteration of the ecological balance was particularly evident from the great population diversity of non-*Saccharomyces* yeast species isolated in the two sets of vinifications with sour rot-affected grapes. At the beginning of vinification, sets with 10% (p/p) and 30% (p/p) sour rot grapes (SR10 and SR30), seven non-*Saccharomyces*

Table 1. *Saccharomyces* and non-*Saccharomyces* populations ($\log_{10}$ CFU mL^{-1}) in fermentation of sound grapes (control set) and sound grapes contaminated with different amounts of sour rotten grapes (2-year study)

| Vintage | Yeast group | Microvinification set | | | | | | | | |
| | | C | | | SR10 | | | SR30 | | |
		BF	MF	EF	BF	MF	EF	BF	MF	EF
2003	*S. cerevisiae*	5.20	7.64	8.19	4.54	8.36	8.05	4.40	8.94	6.64
	Oxidative or weakly fermentative*				1.53	3.52		1.96	3.39	0.82
	Issatchenkia terricola				2.40			2.49		
	Issatchenkia occidentalis								2.76	
	Zygoascus hellenicus				2.04			2.26		
	Zygosaccharomyces bailii				1.60	2.30		2.78	3.15	5.00
	Zygosaccharomyces bisporus								2.18	
	Total yeast counts	5.20	7.64	8.19	4.55	8.36	8.05	4.42	8.94	6.65
2004	*S. cerevisiae*	5.95	8.25	8.01	5.88	8.26	8.23	5.81	8.32	8.10
	Oxidative or weakly fermentative	1.70	4.09		5.82	6.25		5.99	7.16	
	Issatchenkia occidentalis				3.48	3.98		3.40	3.00	
	Zygoascus hellenicus				2.78	2.52		3.59	4.33	
	Zygosaccharomyces bailii				4.72	3.93		4.38	4.16	2.48
	Basidiomycetes	3.38	3.04		3.45	2.48		3.38	2.83	
	Total yeast counts	5.96	8.25	8.01	6.17	8.26	8.23	6.22	8.35	8.10

*Oxidative yeasts include: *Candida stellata/zemplinina*, *Candida diversa*, *Candida vanderwaltii*, *Pichia galeiformis* and *Pichia membranifaciens*. Weakly fermentative yeasts include: *Hanseniaspora osmophila* and *Hanseniaspora uvarum*.
BF, beginning fermentation; MF, middle fermentation; EF, end fermentation; C, control set (100% sound grapes); SR10, 10% (p/p) sour rot grapes; SR30, 30% (p/p) sour rot grapes.

species were detected (2003 and 2004 vintages). *Zygoascus hellenicus*, *Issatchenkia* spp. and *Z. bailii* were always isolated in both vintages. During the initial and middle fermentation phases, in the 2004 vintage, the oxidative or weakly fermentative yeasts *C. stellata/zemplinina* and *H. uvarum* were the most frequent yeast species isolated from SR10 and SR30 microvinification sets. In middle fermentation, a higher number of species was detected in the trial with a greater proportion of affected grapes in 2003. Considering basidiomycetous yeasts, in 2004, they were recovered in numbers ranging from 2.48 to $3.38 \log CFU\,g^{-1}$ from beginning and middle fermentations. In both vintage years, only *Z. bailii* persisted in all stages of sour rot-affected fermentations.

At the end of fermentations, *S. cerevisiae* was the sole recovered species from the trial with 10% sour rot grapes, while *Z. bailii* reached $5.00 \log CFU\,mL^{-1}$ in 2003, and $2.48 \log CFU\,mL^{-1}$ in 2004, with 30% of affected grapes. The species *P. galeiformis* was only detected during the middle and end of fermentation in the 2003 vintage, with 30% of grapes affected by sour rot.

Natural yeast flora from sound and sour rotten grapes at harvest time in 2004

Table 2 shows the ascomycetous species recovered from one sample of 100 g of healthy and sour rotten berries from the 2004 harvest, as well as the health status and culture media utilized. Significant differences in the health of grapes between yeast populations were found. The mean total yeast population counts and species diversity in sour rotten grapes were higher than those of healthy grapes. In sound grapes, *C. stellata/zemplinina*, *H. uvarum* and *L. thermotolerans* populations were identified, with *L. thermotolerans*

Table 2. *Ascomycetes* enumeration ($\log CFU\,g^{-1}$) in 100 g samples of sound and sour rot damaged grapes at harvest time (2004 vintage) as a function of isolation culture media

Yeast species	Sound grapes		Sour rot grapes	
	Medium	Count	Medium	Count
Basidiomycetes	–	ND	DBDM	3.86
Candida stellata/zemplinina	GYP	4.00	GYP	6.34
Hanseniaspora uvarum	DBDM	3.70	GYP	6.26
			DBDM	4.75
Issatchenkia terricola	–	ND	GYP	6.62
			ZDM	4.88
Lachancea thermotolerans	GYP	4.54	GYP	6.34
Zygoascus hellenicus	–	ND	DBDM	3.82
Zygosaccharomyces bailii	–	ND	GYP	7.00
			ZDM	7.65
Total yeasts	GYP	4.65	GYP	7.31
	DBDM	3.70	DBDM	4.80
			ZDM	7.65

ND, not detected.

being the most frequent species. These three ascomycetous yeasts were also isolated in damaged grapes. The species *Z. bailii* ($7.65 \log CFU\,g^{-1}$), *I. terricola* ($6.34 \log CFU\,g^{-1}$) and *Z. hellenicus* ($3.82 \log CFU\,g^{-1}$) were only recovered from 100% sour rotten berries sample. The latter species was only recovered in DBDM selective culture media. Surprisingly, no basidiomicetous yeasts were detected from the healthy grape samples, while $3.86 \log CFU\,g^{-1}$ were detected from sour rot-damaged grape samples.

Concerning culture media, the highest counts were obtained with GYP for all species, except *Z. bailii*. The ZDM selective medium provided higher counts for *Z. bailii* than GYP in sour rotten grapes.

Yeast population dynamics during the sour rot ripening process

In 2005, the study of yeast population dynamics during the grape-ripening period was performed in samples of sound and sour rot-affected bunches collected from the same vine sets during three different phases, from veraison until harvest time. Table 3 shows the results of the different yeast populations isolated in the three ripening phases. Since the beginning of the rotting process, sour rot-damaged grape samples always exhibited higher total yeast counts and a much greater diversity of yeast species.

In sound grape samples, during the entire ripening period, basidiomycetous species dominated the yeast populations. A total of four ascomycetous yeast species were isolated from sound grapes at the middle of ripening the phase (vine set1 and vine set2) and before the harvest (vine set1), in counts up to $2.70 \log CFU\,g^{-1}$. *Candida stellata/zemplinina* ($2.61 \log CFU\,g^{-1}$ in vine set1 and $1.70 \log CFU\,g^{-1}$ in vine set2), *H. uvarum* ($1.88 \log CFU\,g^{-1}$ in vine set1) and *P. membranifaciens* ($1.18 \log CFU\,g^{-1}$ in vine set1 and $1.48 \log CFU\,g^{-1}$ in vine set2) were recovered from sound grapes at the middle phase and *C. vanderwaltii* ($1.00 \log CFU\,g^{-1}$ in vine set1) at the harvest time (Table 3).

Concerning sour rot samples, 19 ascomycetous yeast species were isolated including the four species present in sound grapes. The 15 yeast species only recovered from rotten grapes during all sampling dates were: *C. amapae*, *C. diversa*, *C. ethanolica*, *C. tropicalis*, *C. oleophila*, *H. guilliermondii*, *H. occidentalis*, *P. guilliermondii*, *P. kluyveri*, *I. occidentalis*, *I. orientalis*, *I. terricola*, *L. thermotolerans*, *Z. hellenicus* and *Z. bisporus* (Table 3). Higher yeast species diversity was detected at the middle of the ripening phase, in which 12 (vine set1) and 11 (vine set2) ascomycetous species were isolated. In this phase, *C. vanderwaltii* ($7.49 \log CFU\,g^{-1}$ in vine set2) and *H. uvarum* ($7.49 \log CFU\,g^{-1}$ in vine set2) were the dominant yeast species. The isolation of spoilage yeasts *P. guilliermondii* ($3.78 \log CFU\,g^{-1}$ in vine set1) and *Z. bisporus* ($4.51 \log CFU\,g^{-1}$ in vine set1) was only achieved

Table 3. Yeast dynamics during grape ripening (2005 vintage)

	Grape ripening phase											
	After veraison				Middle of ripening				Before the harvest			
	Vine set1		Vine set2		Vine set1		Vine set2		Vine set1		Vine set2	
Yeasts	S	SR	S	SR	S	SR	S	SR	S	SR	S	SR
Basidiomycetes	2.81	5.54	2.38	5.70	2.11	5.01	1.90	5.23	1.90	4.21	2.80	4.39
Candida amapae				5.70		3.95		4.92				5.65
C. diversa				5.70								
C. ethanolica				4.11								
C. oleophila								2.08				
C. stellata/C. zemplinina		5.00			2.61		1.70					
C. tropicalis												2.90
C. vanderwaltii				6.74		6.82		7.49	1.00	6.58		7.11
Hanseniaspora uvarum				6.60	1.88	6.08		7.08				6.30
H. guilliermondii		6.08		6.08		4.68		4.76		6.18		5.70
H. occidentalis		5.30										
Pichia guilliermondii						3.78						
P. kluyveri		2.48		3.04		4.11		5.08				
P. membranifaciens		6.18			1.18	5.48	1.48	5.70		5.95		4.70
Issatchenkia occidentalis						1.88		3.72				
I. orientalis				6.48		5.30						5.30
I. terricola				6.00				4.40				3.00
Lachancea thermotolerans		4.70		6.00		5.30		6.00		5.00		6.00
Zygoascus hellenicus		3.61		1.40		3.58		4.28		1.90		
Zygosaccharomyces bisporus						4.51						
Total *Ascomycetes*		6.48		7.22	2.70	6.94	1.90	7.65	1.00	6.80		7.24
Total yeast counts	2.81	6.53	2.38	7.24	2.80	6.94	2.20	7.65	1.95	6.80	2.80	7.24

Results reported as log CFU g^{-1}.

S, sound grapes; SR, sour rot damaged grapes.

during middle ripening in vine set1 (Table 3). In this vintage year, *Z. bailii* was not recovered in any grape-ripening phase.

Despite the increased species diversity since the beginning of the rotting process, the yeast diversity was slightly lower at harvest time (five ascomycetous species in vine set1 and nine ascomycetous yeast species in vine set2) which suggests that the final stage provides a more restrictive environment. In addition, results show that the diversity of isolated species was dependent on the vine set (Table 3). For example, if results from both vine sets are pooled, 14 different species were isolated during the first ripening phase (after veraison). However, the diversity reduced to seven species (vine set1) or 11 species (vine set2) if only one vine set was analysed.

Overall yeast species dissemination in sound and sour rotten grapes

In ecological terms, dissemination studies should include a large number of samples, over several years. Furthermore, the detection and isolation of a species is more ecologically relevant than its quantification (Lachance, 2003). Therefore, the overall species identified over a 3-year period is presented in Table 4. In this study, *C. stellata/zemplinina*, *I. occidentalis* and *Z. hellenicus* were isolated during 2003, 2004 and 2005 vintages. *Candida stellata/zemplinina* was recovered from both healthy and sour rot grapes, while *I. occidentalis* and *Z. hellenicus* were only isolated from sour rotten grape samples.

Beyond the presence of basidiomycetous yeasts, only five ascomycetous species (*C. stellata/zemplinina*, *C. vanderwaltii*, *H. uvarum*, *P. membranifaciens* and *L. thermotolerans*) were isolated from sound grape samples and always recovered with GYP general-purpose media (Table 4). Regarding sour rot grapes, a much higher diversity was found. A total of 22 different ascomycetes were isolated in this ecological study, from which only 17 species were present in these grapes. Therefore, this observation indicates that the typical grape yeast flora is greatly changed when grapes are damaged by sour rot. In our study, these different species were *C. amapae, C. diversa, C. ethanolica, C. tropicalis, C. oleophila, H. guilliermondii, H. occidentalis, H. osmophila, P. galeiformis, P. guilliermondii, P. kluyveri, I. occidentalis, I. orientalis, I. terricola, Z. hellenicus, Z. bisporus* and *Z. bailii*. This greater diversity was particularly evidenced by the use of several culture media. The general-purpose medium GYP enabled the recovery of the highest number of ascomycetous species

Table 4. Non-*Saccharomyces* species populations on healthy and sour rot grapes and influence of culture media on species recovery (3-year study)

Yeast group	Yeasts	Vintage year	Sound grapes	Sour rot grapes	Culture media			
					GYP	MRS	DBDM	ZDM
Basidiomycetes		2003, 2004, 2005	+	+	+		+	
Oxidative or weakly fermentative	*Candida amapae*	2005		+	+		+	
	C. diversa	2004, 2005		+				+
	C. ethanolica	2005		+				+
	C. oleophila	2005		+			+	
	C. stellata/C. zemplinina	2003, 2004, 2005	+	+	+			
	C. tropicalis	2005		+			+	
	C. vanderwaltii	2003, 2005	+	+	+			
	Hanseniaspora uvarum	2004, 2005	+	+	+		+	
	H. guilliermondii	2005		+	+		+	
	H. occidentalis	2005		+	+			
	H. osmophila	2003		+		+		
	P. galeiformis	2003		+				+
	Pichia guilliermondii	2005		+			+	
	Pichia kluyveri	2005		+				+
	P. membranifaciens	2003, 2005	+	+				+
Issatchenkia spp.	*I. occidentalis*	2003, 2004, 2005		+	+			+
	I. orientalis	2005		+	+			+
	I. terricola	2003, 2005		+	+	+		+
Zygoascus hellenicus		2003, 2004, 2005		+			+	
Zygosaccharomyces related species	*Lachancea thermotolerans*	2004, 2005	+	+	+			
	Zygosaccharomyces bailii	2003, 2004		+	+			+
	Z. bisporus	2003, 2005		+				+
Total ascomycetous species			6	23	11	2	8	10

(10). However, the additional three culture media provided 10 other ascomycetous species. In particular, ZDM selective culture media were crucial for the isolation of technologically relevant *Z. bailii* and *Z. bisporus* species. The isolation of *C. oleophila*, *C. tropicalis*, *P. guilliermondii* and *Z. hellenicus* was only possible because of the use of DBDM selective culture media (Table 4).

Discussion

The impact of damaged grapes on yeast diversity and their influence on yeast dynamics during alcoholic fermentation were studied during three consecutive vintages. Our results are in accordance with the literature showing the predominance of basidiomycetous, apiculate and oxidative or weakly fermentative species in grapes (Pretorius, 2000; Fleet, 2003). The influence of grape health on yeast ecology demonstrated increased yeast numbers and diversity in damaged grapes and in their respective fermentations. During the three vintages, five ascomycetous species were isolated from sound grapes, while 17 ascomycetous species were present only in damaged samples.

In technological terms, our most significant result was the isolation of *Z. bisporus* (2003 and 2005 vintages) and the osmotolerant and acidophilic species *Z. bailii* (2003 and

2004 vintages), which is regarded as one of the most dangerous wine spoilage species (Loureiro & Malfeito-Ferreira, 2003). The frequency of isolation of *Z. bisporus* from foods is much lower than that of *Z. bailii*, although it has a similar capacity to cause food spoilage and is preservative resistant (Pitt & Hocking, 1999). Jolly *et al.* (2003) reported the rare isolation of *Z. bailii* and *Zygosaccharomyces* spp. from sound Chardonnay grapes, while Nisiotou *et al.* (2007) isolated *Z. bailii* from fermentations of *Botrytis*-affected grapes. González *et al.* (2007) isolated *Z. bisporus* at the end of only one wine fermentation set from a total of 14 red grape must fermentations in Tenerife. *Zygosaccharomyces bisporus*, isolated from sherry film, was shown to resist sorbic acid and sulphur dioxide (Splittstoesser *et al.*, 1978), and to produce odorous acyloins in sherry wines (Neuser *et al.*, 2000). However, it is not usually regarded as a dangerous wine spoilage yeast (Loureiro & Malfeito-Ferreira, 2003).

Other species, less relevant in technological terms than *Zygosaccharomyces* spp., were also detected. *Lachancea thermotolerans* was isolated from sound and damaged grapes. The *Lachancea* genus has been classified recently as a result of closely related DNA sequences between the species *Z. fermentati*, *Z. cidri*, *K. thermotolerans* and *K. waltii* (Kurtzman, 2003). Baleiras-Couto *et al.* (2005) reported the

isolation of *K. thermotolerans* species at the middle of several red musts fermentations, and Xufre *et al.* (2006) showed its isolation from white grape juices. *Lachancea thermotolerans* has been detected in grapes affected by honeydew (Barata *et al.*, 2008) and from *Botrytis*-affected grape musts (Nisiotou *et al.*, 2007). *Zygosaccharomyces fermentati* (or *Z. cidri*) has been reported in the initial step of sherry fermentation at the winery level (Esteve-Zarzoso *et al.*, 2001) and at the end of laboratory grape juice fermentation obtained by winery crushing of grapes (Romano & Suzzi, 1993). This species has the ability to produce volatile compounds in synthetic sherry-like medium (Freeman *et al.*, 1977). To the best of our knowledge, there are no reported wine spoilage outbreaks because of the *Lachancea* clade. However, the affinity with *Zygosaccharomyces* spp. justifies the future investigation of any possible spoilage ability. Another species, *Z. hellenicus*, or its anamorph, *C. steatolytica* (Smith *et al.*, 2005), has seldom been described as a grape contaminant. It has been isolated from Riesling grapes in Ontario (Holloway *et al.*, 1990) without any particular technological significance (Chamberlain *et al.*, 1997). Guerzoni & Marchetti (1987) sporadically isolated this species from sour rot-damaged grapes and not from sound grapes. Because of the utilization of the DBDM selective media, we have shown its frequent isolation previously (Barata *et al.*, 2008). We are not aware of wine spoilage caused by this species, but it would be interesting to understand why it is commonly isolated in damaged grapes. The recovery of *P. guilliermondii* was also only achieved using DBDM medium. This species (or its anamorph *Candida guilliermondii*) is regarded as a rare contaminant of grapes and musts (Jolly *et al.*, 2003). We have already isolated it from grapes in the vineyard, grape musts, grape stems, wines and insects using DBDM (Dias *et al.*, 2003; Martorell *et al.*, 2006; Barata *et al.*, 2008). Recently, *P. guilliermondii* was isolated from the beginning and the end of healthy fermenting musts from Greece (Nisiotou *et al.*, 2007). Therefore, this species seems to be a common inhabitant of winemaking environments, although with low frequency. It has the ability to produce 4-ethylphenol in grape musts before fermentation, but does not spoil wines by volatile phenol production (Barata *et al.*, 2006).

The yeast dynamics study performed during grape ripening (2005) showed that in healthy grapes, basidiomycetes and *C. stellata/zemplinina* were the most frequent species, while in sour rotten grapes *H. uvarum* and *C. vanderwaltii* dominate the yeast populations in all three grape-ripening phases. Other common isolates belong to the genera *Candida* and *Pichia*, which are present in both types of health states. These species are common contaminants of grapes (Fleet, 2003), so their isolation was not unexpected. The distinction between *C. stellata* and *C. zemplinina* has been described recently (Sipiczki, 2003; Sipiczki *et al.*, 2005) and was not performed because we could not recover the strains

from the maintenance slants, probably because of acetic acid production (Sipiczki, 2004). Probably, our strains were also *C. zemplinina* because it was found more frequently than *C. stellata* in damaged grapes used for botrytized wines (Csoma & Sipiczki, 2008). These genera are not regarded as dangerous wine spoilers (Loureiro & Malfeito-Ferreira, 2003).

In the microvinification trials, results showed that several yeast species were present during the beginning and middle fermentation phases, emphasizing the diversity of microorganisms that constitute the fermentation environment. A greater diversity of yeast species at initial fermentation phases was clearly observed when sour rot-damaged grapes were introduced in the vinification. Most of the recovered species from microvinifications with sour rotten grapes are regarded as innocuous to wine stability and do not survive fermentation with the addition of *S. cerevisiae*. The most relevant result of our work was the consistent recovery of the species *Z. bailii* during all fermentation steps with 30% (p/p) sour rot-damaged grapes (2003 and 2004 vintages). This demonstrates that *Z. bailii* from sour rot grapes is an important contamination source of wineries and wines. If technological preventive measures fail, this species may contaminate winery surfaces and may be hazardous to bottled wine stability.

The current application of direct molecular methods for ecological analysis has revealed a great diversity of microorganisms in various habitats that were not detected previously by plating methods (Pace, 1997). Recently developed direct molecular techniques are useful to detect the presence of different species (Prakitchaiwattana *et al.*, 2004). However, these are not sensitive enough to recover minority species and are therefore not able to detect minor but technologically significant differences in the ecology of damaged grapes. The results of this work, and those of Barata *et al.* (2008), show that in order to ascertain the extent of the impact of damaged grapes on yeast diversity, the use of selective media directed to the recovery of minority species is required, as hypothesized by Loureiro *et al.* (2004). This fact was already demonstrated in early ecological works on wine environments (Van der Walt & van der Kerken, 1961; Davenport, 1974; Florenzano *et al.*, 1977) but has not been taken into account in recent studies that rely on the utilization of general-purpose media, followed by species identification by molecular methods.

In conclusion, our results strongly indicate that grape damage constitutes the main factor determining the yeast ecology of grape surfaces. The alteration of the ecological balance is particularly evidenced by the high frequency of isolation of *Z. hellenicus* and *Issatchenkia* spp., only from sour rotten grape samples. Furthermore, the scarce but technologically relevant isolation of *Z. bisporus* and *Z. bailii* shows the importance of using selective culture media. The

latter species, carried into the winery by damaged grapes, endure fermentation and may contaminate wine during the next stages of winemaking.

Acknowledgements

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