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Biodiversity in yeast populations associated with botrytised wine making

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Botrytised wines are made from grapes infected by Botrytis cinerea *(noble rot). During the rotting process a number of important transformations occur that have a large impact on the behaviour of the fermentation and on the organoleptic qualities of the finished wine. Here we briefly review what is known about the yeasts that colonise the rotting berries and act during fermentation and ageing of the botrytised wine. Highly diverse yeast populations are present in each phase. The major yeasts of fermentation,* Saccharomyces cerevisiae, S. uvarum *and* Candida zemplinina *show high intraspecies diversity in physiological properties and genome structure. Their simultaneous persistence throughout the fermentation process is due to the complementary physiological properties, for example in preference for fructose and glucose.*
Key words: noble rot, botrytis, Saccharomyces, Candida, fructophilic yeast

Biodiversität der Hefepopulationen im Zusammenhang mit der Herstellung von Botrytisweinen. *Botrytisweine werden aus Trauben hergestellt, die von Botrytis cinerea (Edelfäule) befallen sind. Während der Entwicklung dieser Edelfäule findet eine Vielzahl von wichtigen Transformationen statt, die einen großen Einfluss auf den Gärverlauf und die sensorische Qualität des fertigen Weines haben. Kenntnisse über die Hefen, die die edelfaulen Trauben kolonisieren und dann bei Gärung und Ausbau der Botrytisweine aktiv sind, werden dargestellt. In jeder Phase findet man Hefepopulationen hoher Diversität. Die für die Gärung wichtigsten Hefen,* Saccharomyces cerevisiae, S. uvarum *und* Candida zemplinina, *zeigen eine hohe intraspezifische Diversität hinsichtlich Genomstruktur und ihrer physiologischen Eigenschaften. Auf Grund ihrer komplementären physiologischen Eigenschaften, z. B. ihrer jeweiligen Präferenz für Fructose oder Glucose, können sie den ganzen Gärprozess nebeneinander überdauern.*
Keywords: Edelfäule, Botrytis, Saccharomyces, Candida, fructophile Hefen

One intriguing phenomenon associated with the infection of grapevine by *Botrytis cinerea* is the ability of this fungus to live both pathogenically and saprophytically on the attacked host. It can cause the crop-destroying grey rot but it can also have desired effects when resulting in noble rot (pourriture noble, Edelfäule) in grapes (Fig. 1). Grey rot (or grey mould) causes heavy losses of yield in wine grapes and is one of the most serious threats for vine growers. Noble rot is a highly specific process resulting in "botrytised" grapes that produce the greatest white wines. The role of *B. cinerea* in this process was described for the first time in Germany almost 120 years ago (Müller-Thurgau, 1888). In this review we briefly summarise what is known about the diversity of yeast populations of the noble rot affected grape and the fermenting and ageing botrytised wine.

Noble rot botrytisation

Noble rot is critically dependent on specific micro-climatic conditions during grape ripening that prevent the invading fungus from causing malevolent destructive rotting. The most important elements of these conditions are the long warm autumn and the fluctuation of humidity (e.g. humid nights followed by dry sunny days). Under these conditions, the penetrating *Botrytis* hyphae launch a complex process that results in beneficial physical and chemical changes in the berry. The growing hyphae consume acids and sugar, drastically reduce the soluble nitrogen compounds, produce glyce-

Fig. 1. Noble rot: desiccation of *Botrytis*-infected berries

rol and various metabolites (Müller-Thurgau, 1888; Dittrich et al., 1974; Doneche, 1993). One physical consequence is the rupture of the skin of the berry which allows that much of the water evaporates leading to a drastic increase of sugar content and the concentration of other substances. The *Botrytis*-generated injuries make the berry accessible to secondary colonisation by other microorganisms which gradually become a tiny ecosystem of interacting filamentous fungi, yeasts and bacteria (Fleet et al., 1984; Joyeux et al., 1984; Duhail et al., 1999; Barbe et al., 2001; Antunovics et al., 2003; Bene and Magyar, 2004; Sipiczki, 2006) that enrich the grape juice with additional aroma and flavour compounds. As fermentative yeasts are also present in the colonising microflora (Antunovics et al., 2003; Bene and Magyar, 2004), fermentation of the grape juice commences already in these berries in the vineyard, long before it is pressed.

Few geographical areas within the European Union and elsewhere in the world are suitable for making botrytised sweet white wines. The "greatest" wines are produced in Sauternes-Barsac (France), Rheingau and Mosel-Saar-Ruwer (Germany), Tokaj (shared by Hungary and Slovakia) and Rust (Austria), but a number of other wine regions in France, Italy, Romania, Austria and Switzerland have climatic conditions that allow at least moderate noble rot. Outside Europe, botrytised wines can be produced in the USA (e.g. Napa Valley and Santa Barbara in California), Canada (Niagara Peninsula), Chile (Valle del Maule), Australia (New South Wales and Victoria) and New Zealand (Te Kauwhata).

The botrytised grape must is a harsh environment for yeast growth and activity

Botrytis invasion and the desiccation of the berries cause drastic changes in the composition of the grape juice (Dittrich, 1989; Doneche, 1993). Although the fungus can metabolise more than a third of the sugar (Dittrich et al., 1974; Sponholz et al., 1987), the final sugar concentration can be as high as 60 to 70 % depending on the extent of botrytisation (proportion of noble rot berries). The high sugar concentration drastically reduces the growth and fermentative activity of yeasts in the must (Müller-Thurgau, 1888). Interestingly, the consumption of sugar (reduction of sugar content) by *Botrytis* is not unanimously positive for the yeasts because usually more glucose is metabolised than fructose (Sponholz et al., 1987) which increases the fructose/glucose ratio in the must. The high fructose/glucose ratio can have inhibitory effects on *Saccharomyces* (Minarik et al., 1978; Gafner and Schütz, 1996). In addition, *Botrytis* also produces agents that directly affect yeast growth and activity. Botryticine (a group of heteropolysaccharides), botrydial, norbotryal acetate and botrylactone have fungistatic activities and can cause fermentation difficulties (Doneche, 1993; Fehlhaber et al., 1974; Cuevas and Hanson, 1977; Weimar et al., 1979). In contrast, Minarik (1983 and 1986) observed an accelerating effect of *Botrytis* extract on the fermentation activity of *S. cerevisiae* (*S. oviformis*). The mechanism of this effect has not been elucidated yet.

The taxonomic diversity of natural yeast flora

Pre-harvest yeast flora. The injuries generated by the penetrating *Botrytis* hyphae serve as a gateway for a secondary invasion of the berry by additional microbes. The secondary colonists can be yeasts coming from the normal surface microflora of the ripening berries and yeasts brought by insects visiting the injuries to feed on the grape juice. These yeasts then rapidly colonise the rotting berries and develop large populations. The major species found in samples of noble rot grapes or

in fresh must produced from botrytised grapes are listed in Table 1. Although certain species (e.g. *Kloeckera apiculate/Hanseniaspora uvarum* and *Candida/Torulopsis zemplinina/stellata*) were standard components of the colonising microflora, the lists of all yeast species present in the samples showed considerable diversity. The differences indicate that the composition and development of the yeast populations caused by over-ripening can be affected by environmental conditions and may also depend on the grape variety and vineyard site (terroir). Studies carried out in vineyards of Sauternes (France) demonstrated that humid climate increased the number of microorganisms on the overripe berries, and that more microorganism were found on berries of the variety 'Sauvignon blanc' than on berries of 'Semillon' (Duhail et al., 1999). An interesting result of these studies is that *Saccharomyces* is not a regular component of the pre-harvest microflora, or its frequency is below the threshold of detectability by the methods used. The fermentative yeasts most probably produce some alcohol in the berries by the time of harvest (Joyeux et al., 1984).

Fermenting yeast flora

The pre-harvest colonists then become the microflora of the fresh must. Since the noble rot berries contain large populations of multiple yeast species, the fermentation starts in the botrytised must with a yeast community of high density and complexity (Fleet et al., 1984). But for most of the yeasts the botrytised must is a rather hostile environment. The apiculate yeasts (*Kloeckera* and *Hanseniaspora*), *Metschnikowia*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus* and most *Candida* species gradually die off (Fleet et al., 1984), making room for the more adaptable *Saccharomyces* populations (Sipiczki, 2010). In spite of being rather sporadic in grapes, *Saccharomyces* takes the lead within a very short time. A characteristic feature of the fermenting botrytised must is the simultaneous presence of two *Saccharomyces* species. Although the major yeast is *S. cerevisiae* like in other wines, *S. uvarum* (*S. bayanus* var. *uvarum*) is usually also present (Minarik and Laho, 1962; Naumov et al., 2000, 2002; Antunovics et al., 2005a; Masneuf-Pomerede et al., 2007). Its proportion can be high and it can even dominate the fermentation (Usseglio-Tomasset et al., 1980; Naumov et al., 2000; Sipiczki et al., 2001). The major non-*Saccharomyces* yeast, which can survive until the end of fermentation, is *Candida zemplinina* (synonym: *Torulopsis*) (Minarik et al., 1978; Cocolin et al., 2001; Mills et al., 2002), a highly fructophilic species (Mills et al., 2002; Magyar and Toth, 2010), which is frequently mistaken for *Candida stellata* and *Starmerella bombicola* (Sipiczki et al., 2005; Csoma and Sipiczki, 2008).

The three major yeasts of botrytised wines (*S. cerevisiae, S. uvarum* and *C. zemplinina*) can probably coexist because their physiological properties are complementary. For example their sugar preferences are different. The wine strains of *S. uvarum* are usually strongly glucophilic (Schütz and Gafner, 1995). Most *S. cerevisiae* wine strains also show preference for glucose (Berthels et al., 2004) but *C. zemplinina* is highly fructophilic (Mills et al., 2002; Magyar and Toth, 2010). Because of these differences they need not compete for the same carbon source and can maintain the favourable balanced ratio between glucose and fructose necessary for the smooth progress of fermentation. It has been shown that the reduction of the glucose/fructose ratios can slow down the process (sluggish and stuck fermentation). In Swiss (non-botrytised) stuck wines Gafner and Schütz (1996) detected fructose levels more than ten times higher than the glucose levels. Considering these observations, the preferential consumption of fructose by the fructophillic *C. zemplinina* in the fermenting botrytised must may have a balancing effect.

A special type of botrytised wine is Essence which is made from selectively harvested botrytised grapes. Its high sugar concentration (up to 60 to 70 %) reduces the activity of *Saccharomyces* and *C. zemplinina* and gives advantage to the osmotolerant species *Zygosaccharomyces bailii*, *Z. rouxii* and *C. lactis-condensi* (Csoma and Sipiczki, 2007 and 2008).

Post-fermentation (ageing and refermentation) yeast flora

Fermentation can be stopped by addition of sulphur dioxide to the wine. For *Botrytis*-affected wines, the maximum level accepted by the OIV is 400 mg/l. Although this concentration inhibits microbial activity, viable yeast cells can be found in the ageing wine (Table 2). These may be survivors from the fermenting population or new colonists. Taxonomic examination classified them to non-*Saccharomyces* species (Table 2). Certain yeasts persist for long periods of time in a "viable but non-culturable"-like state (Divol and Lonvoud-Funel, 2005). These can cause a refermentation of the sweet wine when the SO_2 level decreases. Pseudohyphal growth on the wine surface is another type of post-fermentation yeast activity in certain types of botrytised wines. In Tokaj wines, *S. cerevisiae* race *capensis* and *S.*

Table 2: Fermentation: Occurrence of yeasts in fermenting botrytised wine

	Region	Species	Remark	Reference
Early stage	Sauternes (F)	*Hanseniaspora uvarum, Torulopsis stellata, Metschnikowia pulcherrima, Candida krusei, Saccharomyces cerevisiae*		FLEET et al., 1984
	Tokaj (H)	*Aureobasidium, Metschnikowia, Rhodosporidium, Rhodotorula, Hanseniaspora, Cryptococcus, Candida zemplinina/stellata*		SIPICZKI et al., 2001; ANTUNOVICS et al., 2003
	California	*Candida zemplinina, Metschnikowia pulcherrima, Kluyveromyces thermotolerance, Hanseniaspora uvarum, H. osmophila*		COCOLIN et al., 2001; MILLS et al., 2002
Main stage	Tokaj (SL)	*Saccharomyces oviformis* (probably *cerevisiae), Candida zemplinina/stellata, Saccharomyces (Zygosaccharomayces) bailii*		MINARIK and LAHO, 1962; MINARIK, 1965, 1969; MINARIK et al., 1978; NAUMOVA et al., 1991; NAUMOV et al., 2002
	Tokaj (H)	*Candida zemplinina, Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces* interspecies hybrid		NAUMOV et al., 2002; SIPICZKI et al., 2001; ANTUNOVICS et al., 2005a
	Tokaj (H)	*Zygosaccharomyces bailii, Z. rouxii, Candida zemplinina, Candida lactis-condensi, Saccharomyces uvarum, Saccharomyces cerevisiae*	Essence wine	CSOMA and SIPICZKI, 2007
	Sauternes (F)	*Saccharomyces cerevisiae* → *Torulopsis stellata (Candida stellata/zemplinina)* → *Hanseniaspora uvarum* → *Metschnikowia pulcherrima* → *Candida krusei* →	Dominating yeast Survived to late stages Died off by day 7 Died off by day 7 Died off by day 7	FLEET et al., 1984
	Sauternes, Barsac (F)	*Saccharomyces uvarum*		NAUMOV et al., 2000
	Sauternes (F)	*Saccharomyces uvarum*		MASNEUF-POMEREDE et al., 2007
	Valpolicella (I)	*Saccharomyces cerevisiae* *Saccharomyces uvarum*		USSEGLIO-TOMASSET et al., 1980; TORRIANI et al., 1999
	California	*Saccharomyces cerevisiae, Candida zemplinina, Kluyveromyces thermotolerance, Hanseniaspora uvarum, H. osmophila*		COCOLIN et al., 2001; MILLS et al., 2002
Post-fermentation*	Sauternes (F)	*Saccharomyces (Zygosacch.) bailii, S. globosus* (probably *uvarum), Pichia membranefaciens, Candida crusei, C. valida*	2 month after fermentation termination	FLEET et al., 1984
	Sauternes (F)	*Saccharomyces cerevisiae, Zygosaccharomyces bailii, Candida stellata/zemplinina, Zygosaccharomyces rouxii*	3 to 9 weeks after fermentation termination	DIVOL et al., 2005
	Sauternes (F)	*Saccharomyces cerevisiae, Zygosaccharomyces bailii, Candida stellata/zemplinina, Rhodotorula mucilaginosa*	Refermentation	DIVOL and LONVAUD-FUNEL, 2005; DIVOL et al., 2006
	California	*Saccharomyces cerevisiae, Candida zemplinina, Kluyveromyces thermotolerance*		COCOLIN et al., 2001; MILLS et al., 2002
	Tokaj (H)	*Saccharomyces cerevisiae* race *capensis* *Saccharomyces cerevisiae* race *aceti*	Floor yeasts	MIKLOS et al., 1994

*In finished and refermenting wine

cerevisiae race *aceti* strains were found that could form pseudomycelium (MIKLOS et al., 1994). Both races are known as typical surface "flor yeasts" essential for the biological ageing of sherry wines (GUIJO et al., 1986). Consistent with the observation made with Tokaj isolates, DIVOL et al. (2005) found flor yeast-type sequences in refermenting *S. cerevisiae* strains isolated in Sauternes.

Diversity and genetic instability of physiological properties

Wine strains of *Saccharomyces* usually ferment glucose faster than fructose, but this is not an absolute rule.

Table 1: Pre-harvest colonisation: occurrence of yeasts on/in *Botrytis*-infected grapes and grape must

Country, region	Species	Reference
South Africa	*Kloeckera apiculata (Hanseniaspora uvarum), Torulopsis stellata (Candida zemplinina/stellata)*	LE ROUX et al., 1973
France, Sauternes	*Kloeckera apiculata (Hanseniaspora uvarum), Torulopsis stellata (Candida zemplinina/stellata), Metschnikowia pulcherrima, Candida krusei, Saccharomyces cerevisiae*	FLEET et al., 1984
Hungary, Tokaj	*Aureobasidium pullulans, Hanseniaspora uvarum, Metschnikowia fructicola, Metschnikowia pulcherrima, Rhodotorula kratochvilovae, Rhodotorula nothofangi, Kluyveromyces thermotolerance, Cryptococcus magnus var. magnus, Candida zemplinina/stellata, Saccharomyces uvarum, Saccharomyces cerevisiae*	BENE and MAGYAR, 2004; ANTUNOVICS et al., 2003; MAGYAR and BENE, 2006; SIPICZKI, 2006; CSOMA and SIPICZKI, 2007
USA, California	*Saccharomyces cerevisiae, Hanseniaspora uvarum, Pichia kluyveri, Metschnikowia pulcherrima, Candida zemplinina*	MILLS et al., 2002

GAYON and DUBOURG (1890) were the first who observed that some "Sauternes" wine yeasts (presumably isolated from botrytised wine fermentation) preferred fructose to glucose. It was proposed that the selective fermentation of fructose by the Sauternes yeast cells resulted from the preferential permeability of their membrane for fructose and from the higher affinity of fructofuranose for hexokinase (GOTTSCHALK, 1946). But no taxonomic identification was presented in the reports; so the yeast studied could have been strains of any of the major species of botrytised wine fermentation.

ANTUNOVICS et al. (2005a) reported on considerable diversity in the physiological properties of *S. uvarum* strains isolated in Tokaj. They found that all physiological tests generally used for the taxonomic differentiation between *S. cerevisiae* and *S. uvarum* were variable. While the type strain of *S. uvarum* assimilated mannitol (Mal^+), melibiose (Mel^+), grows on vitamin-less medium and is sensitive to 37 °C, they found Mal^+ and Mal^-, Mel^+ and Mel^-, vitamin-requiring and vitamin-prototrophic, temperature sensitive and temperature tolerant strains among the *S. uvarum* isolates. MASNEUF-POMAREDE et al. (2010) using the same Tokaj isolates and certain Sauternes strains detected significant diversity in fermentation power, residual sugar level and the production of certain metabolites.

Both *S. cerevisiae* and *S. uvarum* are sexual species. Their cells are capable of dividing by meiosis (sporulation) and fusing by conjugation (sexual fusion of haploid cells). Conjugation can bring different genomes together resulting in heterozygous hybrids. Later, when the cells sporulate, the genome of the hybrid segregates producing spores of diverse recombinant genomes and phenotypes. Each germinating spore forms a clone of vegetatively propagating cells which can mate with each other or with cells of other clones. To study the segregation in natural yeasts of botrytised wine fermentation, *S. cerevisiae* and *S. uvarum* isolates from Tokaj wines were subjected to tetrad analysis (SIPICZKI et al., 2001). Spores were isolated from dissected asci (tetrads), and the physiological properties of the clones produced by them were compared. Practically each spore differed from the parental isolate and from its sister spores in the production levels of secondary metabolites. The segregation patterns indicated that the isolates were heterozygous and the production of the metabolites was under polygenic control. Although sporulation rarely occurs during fermentation, it can take place upon its completion, when starvation switches on the meiosis-sporulation developmental pathway. Due to meiotic segregation of the genomes, the spores produced after fermentation can be much more diverse in genotype and phenotype than their parental diploid cells and can significantly increase the diversity of the yeasts that survive until the next vintage season (SIPICZKI, 2010).

Molecular diversity of the fermenting yeasts

Intraspecies diversity has also been found at molecular level in each of the three major yeast species. Electrophoretic karyotyping detected chromosome size variability. In *C. zemplinina*, two of the three chromosomes varied in size (SIPICZKI, 2004). In the *Saccharomyces* species, the genomes of which are partitioned in higher numbers of chromosomes, the karyotypes were more polymorphic. When yeasts from botrytised Tokaj wines were studied, the *S. cerevisiae* isolates were much more variable in size and number of chromosomal bands than the *S. uvarum* isolates (SIPICZKI et al., 2001; NAU-

MOV et al., 2002; ANTUNOVICS et al., 2003 and 2005a). The *S. cerevisiae* strains isolated from refermenting Sauternes wines were also polymorphic in karyotypes (DIVOL et al., 2005). Similar differences between *S. cerevisiae* and *S. uvarum* were also observed in strains of non-botrytised wines (CSOMA et al., 2010). The higher plasticity of the *S. cerevisiae* chromosomal sets can be one of the factors that make this yeast more adaptable to the drastic changes of the environment taking place during the fermentation process (SIPICZKI, 2010).

Mobile genetic elements may be responsible for at least part of the size polymorphism (RACHIDI et al., 1999) of chromosomes because their sequence identity provides possibilities for recombination between non-homologous chromosomes or between different sites within the same chromosome. The Ty elements are retrotransposons flanked by terminal delta sequences. Each Ty is flanked by a pair of delta sequences, but solo delta sequences also occur in the genomes. The number and location of the delta sequences are variable in the natural wine strains of *S. cerevisiae*. The variability is high enough for differentiation between strains by amplifying their "interdelta" sequences. It has been found that wine strains of identical karyotypes can present different interdelta profiles (DIVOL et al., 2006). The natural wine strains of *S. uvarum* do not have Ty elements and delta sequences. The lack of these sequences as potential recombination sites may account for the lower karyotype diversity in *S. uvarum*.

Another molecular method of identifying genetic differences between *Saccharomyces* strains is based on the comparison of the size of their microsatellites. The recent application of this method to the characterisation of French *S. uvarum* strains isolated from botrytised wine fermentation demonstrated that microsatellite analysis can detect differences even when the karyotypes are identical (MASNEUF-POMAREDE et al., 2007).

The analysis of the 26S rDNA and the ITS1-5.8S rDNA-ITS2 loci revealed an interesting heterozygosity in strains isolated from refermenting Sauternes wines (DIVOL et al., 2006). Their genomes had sequences of standard wine strains and somewhat different sequences characteristic of flor yeast strains involved in sherry fermentation. In addition, these strains exhibited higher expression for the SSU1 gene (coding for sulfite pump) which is also over-expressed in flor strains (DIVOL et al., 2006).

Due to their close phylogenetic relationship, *S. cerevisiae* and *S. uvarum* are interfertile. The cells of their wine strains can conjugate and form interspecies hybrids (SIPICZKI, 2008). Natural hybrids occur in spontaneously fermenting botrytised must (NAUMOV et al., 2002; ANTUNOVICS et al., 2005a; LE JEUNE et al., 2007) and can also be created in laboratory by mating cells or germinating spores (ANTUNOVICS, 2005b). The hybrids can have properties different from those of the parental species. MASNEUF et al. (2002) reported on hybrids that released high amounts of volatile thiols without producing excessive amounts of (beta)-phenylethyl alcohol and its acetate. Meiosis of the alloploid genome results in recombinant spores with composite genomes containing genes from both species (chimeras) (ANTUNOVICS et al., 2005b) which further increase the diversity in the fermenting yeast populations. This occurs when the hybrid has an allopolyploid genome. When the genome is only allodiploid, the viability of the spores is very low. Six natural hybrids from the Alsace area of France were characterised in detail and found to have allodiploid genomes (LE JEUNE et al., 2007).

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