Selective value of QoI-resistant strains of downy mildew (*Plasmopara viticola*)

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Introduction

Plasmopara viticola, the causal agent of downy mildew of grapevine, is responsible for extensive damage in vineyards. At present, chemical control remains the most effective form of management.

Many efficient molecules have been developed, but resistant pathogen populations emerged regularly and rather quickly, one example being general resistance reported to QoI fungicides (Gisi *et al.*, 2002; Magnien *et al.*, 2003). Preventing and delaying resistance adaptation to fungicides in pathogen populations is a major goal in sustainable plant-pathogen management. To manage fungicide resistance phenomena, it is important to understand how resistant populations appear, spread and survive.

A major issue understanding the resistance process is to measure pathogen fitness, i.e. the selective value of resistant and sensitive strains with or without fungicide pressure. The ability of population to spread resistant genotypes and their ability to infect host plants and survive can be evaluated with various methods. Several studies have focused on the fitness of human pathogen bacteria (Andersson, 2006). In such cases, the major point studied concerns individual contribution to the next generation gene pool by measuring the growth rate, with the aid of a Malthusian fitness index (Newton *et al.,* 1997; Kato *et al.*, 1997; Pringle and Taylor, 2002). One difficulty is that filamentous fungi exhibit different types of reproduction in their life cycle: an asexual phase during the growing season with clonal reproduction and a sexual phase with heterothallic or homothallic reproduction. During the clonal phase, fitness may be measured as with bacteria by measuring the quantity of propagules produced from one initial propagule. Nonetheless, other parameters such as the ability of infection or the time necessary to achieve new sporulation.

In this study, we measured fitness parameters (e.g. sporangia size, infection efficiency, infection frequency, sporulation) under controlled conditions for fungicide-resistant and sensitive strains collected from French (35 strains) or German (1 strain) vineyards. The fungicide-resistant strains tended to exhibit fitness that was at least as good as that in fungicide-sensitive strains in laboratory conditions. On the basis of these data, we propose a fitness index for allows classifying strains depending on their selective value. We suggest that, unlike artificial mutations, mutation phenomena under natural selection pressure tend to be selected in high-fitness strains, leading to rather fit resistant strains.

Material and Methods

Isolates

Isolates were maintained on detached leaves placed on water-saturated filter paper in Petri dishes at 21 °C. All experiments were performed with detached leaves or leaf disks of 4 weeks old plants of Cabernet-Sauvignon grown in greenhouses under complementary artificial lighting (photoperiod of 16 h), with 60 to 70% hygrometry and at 21 ± 2 °C. Details on the location and date of collection are given in table 1.

Table 1 - Date and location of QoI-resistant and sensitive strains. Eighteen sensitive strains and 18 QoI-resistant strains of *Plasmopara viticola* were collected from French and German (italic) vineyard.

Strain	Origin	Year	QoI-resistance
AIR07	Beaucaire (30)	2005	S
AVI02	Avize-Epernay (51)	2003	S
BOM06	Bommes (33)	2003	S
CAZ22	Cazaugitat (33)	2003	S
CGU20	Château Guiraud (33)	2003	R
COL	Colmar	2006	S
COU15	Petit Couhins (33)	2003	S
COU15'	Petit Couhins (33)	2003	S
EAU11	Eauze (32)	2005	R
EAU13	Eauze (32)	2005	R
EAU13'	Eauze (32)	2006	R
EAU14	Eauze (32)	2005	R
EPE09	Epernay (51)	2003	S
EPE45	Epernay (51)	2003	S
FEM03	Puligny-Montrachet (21)	2003	S
FRE01	Freibourg	2003	R
EAU08	Eauze (32)	2005	R
LAN123b	(26)	2005	S
LAN127a1	Manciet (32)	2005	R
MIC103	Fleurie (69)	2004	S
MIC123	(26)	2004	S
MIC125	Millet (32)	2005	R
MIC126	Maisonneuve (32)	2005	R
MIC127	Manciet (32)	2005	S
MIC128	Castillon (32)	2005	R
MIC64	Saint Bris (89)	2004	S
MOR08	Morizès (33)	2003	R
MOR22	Morizès (33)	2003	S
PAR29	Parempuyre (33)	2003	R
PAU32	Pauillac (33)	2003	R
PIC21	Latresne (33)	2003	S
PIC59	Couhins (33)	2003	R
ROF01	Rouffignac (17)	2005	S
ROF02	Rouffignac (17)	2005	R
SAL19	Salleboeuf (33)	2003	R
ZAN06	Rauzan (33)	2003	R

The fungicide

The fungicide used belongs to acetamide family or quinone outside Inhibitors (QoI). It inhibits the cytochrome b involved in the electron transfer of the mitochondrial respiration process (Bartlett *et al.*, 2002).

Fitness components

The following fitness components were compared in sensitive and resistant strains: 1) sporulation capacity = number of sporangia produced from a inoculation droplet of 15 µL containing 4,000 sporangia per mL at 7 days postinoculation; 2) infection frequency = proportion of infected points out of

45 points inoculated at 7 days postinoculation; 3) latency period = period necessary to obtain 50 and 100 % of sporulation intensity; 4) sporangia size; 5) infection efficiency = rate of germinated zoospores in stomata versus amount of zoospores not germinated or germinated outside stomata. Malthusian fitness measures the growth rate of a population, $M = \ln(N_t/N_0)/t$, where N_t the number of sporangia at 7 days, N_0 number of sporangia at baseline.

A complex index of fitness (F_{IC}) was also calculated as:

$F_{IC} = S \times L \times M \times IF \times IE$

(**S** is the size of sporangia in µm, **L** is indexed latency time to obtain 50% of total sporulation, **M** is Malthusian fitness, **IF** is the infection frequency and **IE** is the infection efficiency).

All data for each fitness component were subjected to an analysis of variance. Means of each isolate within sensitivity groups and means among sensitivity groups were compared using Duncan's multiple range test at $P = 0.05$.

Results

To assess the competitive fitness components during asexual cycle and in laboratory conditions, we performed the measurements described above. First, we measured the average size of sporangia (data not shown) and found that the average size of QoI-resistant (17.2 ± 2.1 µm) and QoI-sensitive (17.0 ± 2.8 µm) strains was not significantly different. Nevertheless, we sporangia size ranged from 8 to 33 µm between the strains, a difference which may have an impact on their fitness. This difference might be due to the number of zoospores contained in sporangia, or to the different sizes of zoospores. Strains with larger sporangia might have an advantage over those with smaller sporangia ones, but the larger sporangia might also be limited in their dispersion, particularly dispersion by wind.

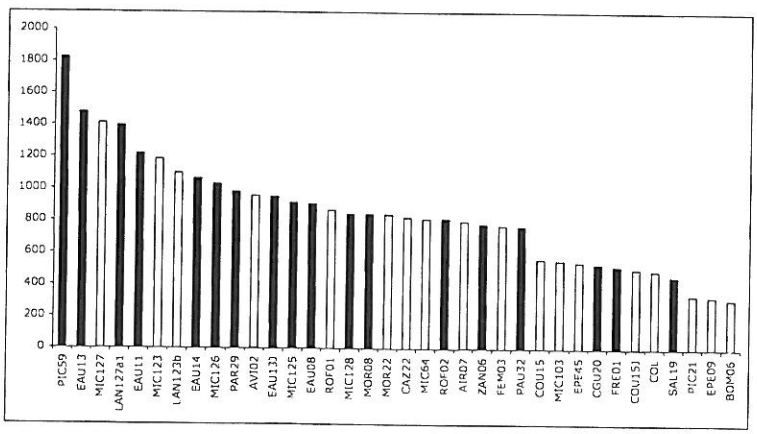

Figure 1 - Sporulation rate of *P. viticola* strains.
The sporulation rate corresponds to sporangia obtained from one inoculated sporangium. The black bars stand for sporulation rate of QoI-resistant strains, and white bars stand for sporulation amount of QoI-sensitive strains.

Then a study was performed to measure the time required to obtain 50 % of total sporulation of each isolate (data not shown). The requirement ranged 89 to 140

hours for strains to reach 50 % of sporulation and the average time was 113 ± 16 hours for both resistant and sensitive strains. The time required to obtain 50 % of sporulation varied about 1.5-fold between the fastest and the slowest strains. Therefore, QoI-resistant and sensitive strains exhibited no significant difference in their latency period.

The third parameter observed was the amounts of sporulation for each of the 36 strains. The results are shown in figure 1. The amount of sporulation varied considerably from one strain to another with a 4.5-fold difference between the strains with the greatest (PIC59 strain) and least (BOM06 strain) sporulation. This indicates that the *P. viticola* strains tested here exhibited different sporulation rates, irrespectively of whether they are QoI-sensitive or QoI-resistant. Resistant strains had an average sporulation equivalent to 960 ± 340 sporangia per inoculated sporangium, while sensitive strains produced only 730 ± 300 sporangia per inoculated sporangium. This difference is significant ($P < 0.05$). QoI-sensitive strains exhibited 1.3-fold less sporulation than QoI-resistant strains, while QoI-resistant strains produced 31 % more sporangia than QoI-sensitive strains. This value is rather high and suggests that resistant strains have an advantage over sensitive strains. This parameter is the closest one to those applied in bacteria fitness studies, where the only parameter considered important is the rate of growth of bacteria strains.

Malthusian fitness was then calculated in order to sort the strains according to their growth rate. The results are shown in table 2. Malthusian fitness calculated as a function of time provides better information about sporulation intensity than sporulation rate alone. The Malthusian index of the 36 strains studied here range from 0.032 (BOM06 strain) to 0.042 (PIC59 strain), exhibiting a 1.28-fold increase for the most prolific strain compared to the least prolific. The average Malthusian index is 0.036 ± 0,002 for sensitive strains and 0.038 ± 0.002 for resistant ones ($P < 0.05$).

Table 2 - Malthusian index and FIC (Complex Index of Fitness) for the QoI-resistant and QoI-sensitive strains of *P. viticola* studied.

Strain	QoI-resistance	Malthus index	F_{IC}
AIR07	S	0,037	0,144
AVI02	S	0,038	0,415
BOM06	S	0,032	0,121
CAZ22	S	0,037	0,167
COL	S	0,034	0,136
COU15	S	0,035	0,068
COU15'	S	0,035	0,366
EPE09	S	0,033	0,102
EPE45	S	0,035	0,225
FEM03	S	0,037	0,233
LAN123b	S	0,039	0,095
MIC103	S	0,035	0,115
MIC123	S	0,040	0,197
MIC127	S	0,040	0,164
MIC64	S	0,037	0,142
MOR22	S	0,038	0,198
PIC21	S	0,033	0,110
ROF01	S	0,038	0,228
CGU20	R	0,035	0,284
EAU11	R	0,039	0,282
EAU13	R	0,041	0,236
EAU13'	R	0,038	0,221
EAU14	R	0,039	0,204
FRE01	R	0,035	0,132
EAU08	R	0,038	0,373
LAN127a1	R	0,040	0,133
MIC125	R	0,038	0,269
MIC126	R	0,039	0,139
MIC128	R	0,038	0,329
MOR08	R	0,038	0,149
PAR29	R	0,039	0,252
PAU32	R	0,037	0,137
PIC59	R	0,042	0,201
ROF02	R	0,037	0,254
SAL19	R	0,034	0,110
ZAN06	R	0,037	0,210

Infection frequency was not significantly different for resistant (IF = 0.96 ± 0.07) and sensitive (IF = 0.90 ± 0.14) strains (P = 0.15) with a dose inoculum of 4,000 sporangia per mL.

The final test performed on QoI-resistant and QoI-sensitive strains was the measurement of infection efficiency. Once again, the range of infection between the strains was high, ranging from 0.24 to 0.70 (2.9 ratio) and indicating the highly variable character of *P. viticola* strains. The average infection efficiency of sensitive strains was 0.42 ± 0.12 and 0.43 ± 0.12 for resistant strains. This difference was not significant (P = 0.78). In conclusion, QoI-resistance phenotype did not correlate with greater or lesser efficiency compared with the sensitive strains.

On the basis of these measurements, we propose an index of fitness where F_{IC} = S x L x M x IF x IE. Table 2 shows F_{IC} values for all strains. This index ranges from 0.07 (COU15) to 0.42 (AVI02). The average value of F_{IC} for QoI-sensitive strains is 0.19 ± 0.09 and the average for QoI-resistant strains is 0.22 ± 0.07. These values are not significantly different (P = 0.34). Therefore, there is no advantage of QoI-resistant strains with F_{IC}, unlike with the Malthusian index. In other words, the cost of resistance is nil or only very minimal. Indeed, the sensitive strains did not exhibit a better F_{IC} than the QoI-resistant strains. Figure 2 shows the F_{IC} values according to which the 36 strains are sorted.

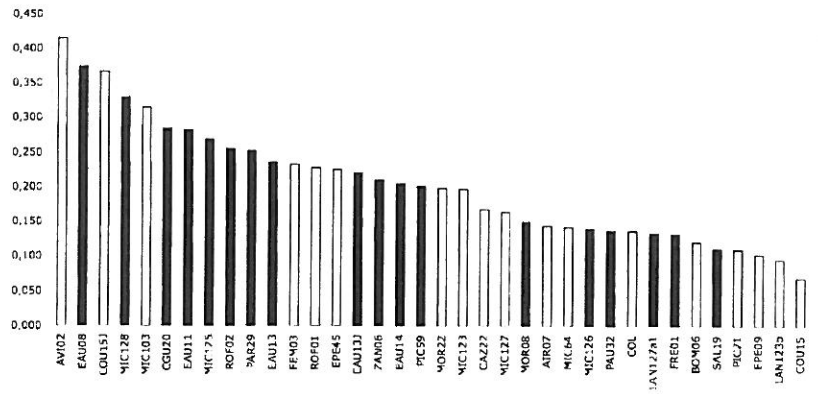

Figure 2 - Complex index of strain fitness (F_{ic}). QoI-resistant strains are represented by solid bars and QoI-sensitive strains are represented by empty bars.

Conclusions and discussion

Some authors (Pringle and Taylor, 2002) consider the asexual phase of filamentous fungi to be like a bacteria type life cycle, so the Malthusian index alone is held to determine the fitness of fungal strains and/or the cost of their resistance. Others consider it is important to take other life cycle parameters in account for fungi. Here, we show that using only the Malthusian index may lead to a very partial view of the problem of fungi fitness. The present findings demonstrate that QoI-resistant strains are fitter than QoI-sensitive ones on the basis of the Malthusian index. However, by using additional parameters and especially infection efficiency, QoI-resistant strains may be shown not to have

any fitness advantage. Therefore, if there is any cost of resistance to QoI, it has no or little influence on final fitness. A putative explanation is that under natural selection pressure, mutation phenomena are exerted on strains with different fitness, but that only very fit strains are selected and survive after mutation. Therefore, in natural conditions, only the most resistant competitive strains after selection pressure, would be detected. In this event, it would be difficult to evaluate the real cost of the resistance acquisition without simulating selection pressure.

The overall fitness of a given population is difficult to predict solely on the basis of fitness components like Malthusian index. For this reason, we attempted to combine fitness components in a manner that approximates overall fitness by calculating the composite fitness index. Similar composite indexes were used by Haymer and Hartl (1983) and Tooley *et al.* (1986).

The data presented here show no difference in fitness parameters between QoI-resistant and sensitive strains originating from natural QoI selection pressure. However, in our experiments, fitness measurements were made in constant environment in growth chambers and did not include other fitness components such as oospore production, overwintering, and oversummering, potentially important under field conditions. To assess our hypothesis that resistant strains are as fit as sensitive strains, we will perform a competition test between resistant and sensitive strains of *P. viticola* with different fitness indexes. By doing this, we will be able to examine whether individual components of fitness in laboratory conditions are good indicators of real fitness of strains.

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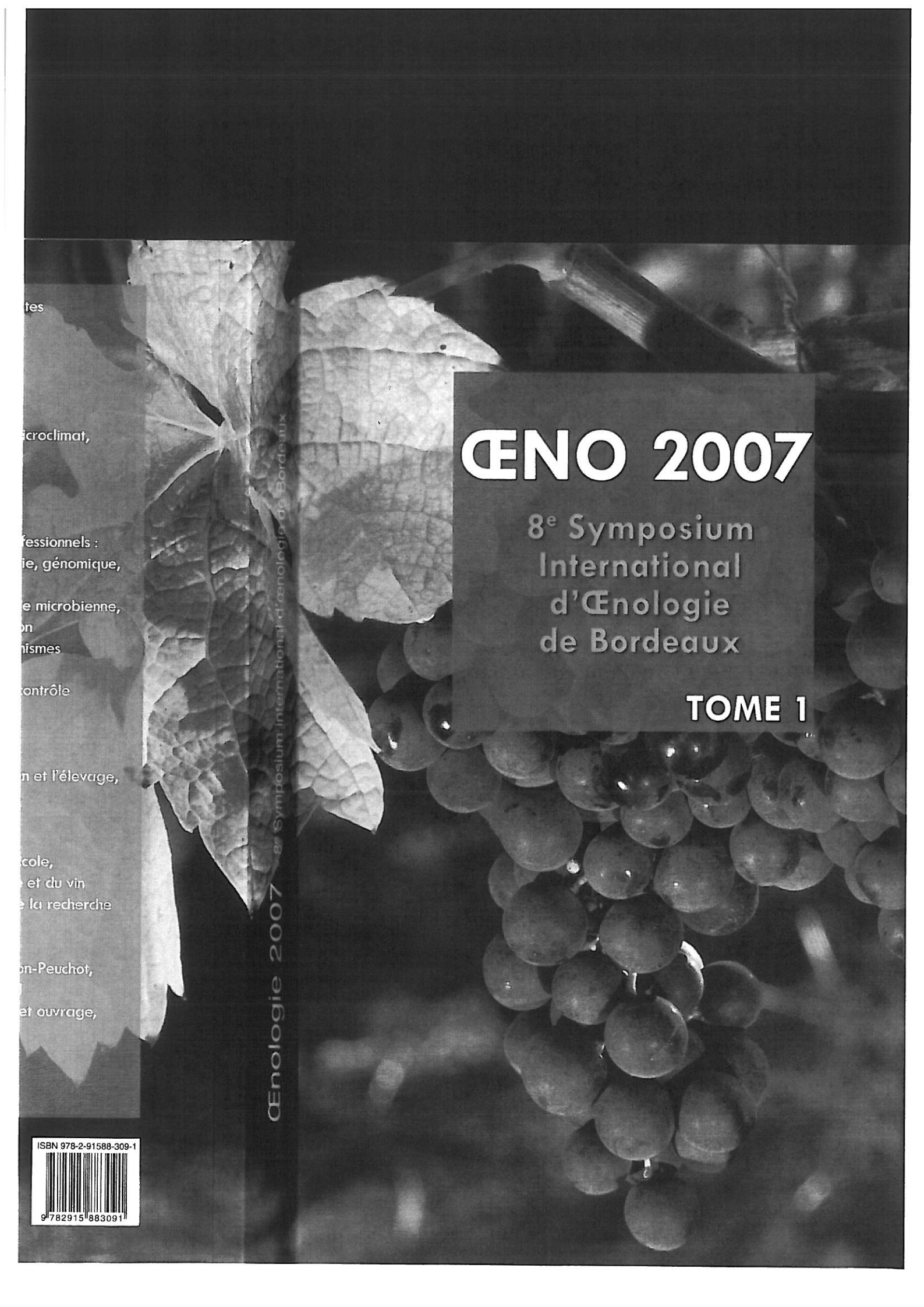

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