Selection for fungicide resistance throughout a growing season in populations of *Plasmopara viticola*

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Abstract A method for evaluating the potential threat of selection for resistance to organically-based fungicides in populations of *P. viticola* is needed to screen a large panel of products alternative to copper in organic viticulture. Populations from an unexposed plot were compared throughout one season with a population sprayed with azoxystrobin (Quadris), reported as engendering selection pressure and resistance, and a population sprayed with an organically-based fungicide (Mycosan). The evolution of the three populations was followed with neutral specific SSR markers and with the specific marker for strobilurin resistance, as control of selection for resistant mutants. A reduction in genetic diversity of the *P. viticola* population was observed in the population sprayed with azoxystrobin, consistent with directional selection toward higher resistance, confirmed by an enhanced frequency of resistant mutants with respect to the unexposed population. In contrast, a higher diversity and a reduced frequency of resistant mutants were observed in the population sprayed with the organically-based fungicide. Assessing a reduction of genotypic diversity allows the detection of selection for resistance and constitutes a valid instrument for screening a large panel of products with non-specific, different and possibly indirect modes of action.

Plasmopara viticola, the causal agent of downy mildew, is considered one of the most important pathogens of grape. This diploid obligate biotroph oomycete affects leaves and fruits of grape plants and causes losses through killing of leaf tissues and defoliation, through production of low quality or entirely destroyed grapes, and through weakening, dwarfing and killing of young shoots. Control of the pathogen is generally achieved with chemical fungicide and copper salt applications (Aziz et al. 2006); organic agriculture depends strongly on the latter. Copper is known as one of the trace elements with the most deleterious effects on living organisms in soil (Renella et al. 2002). Harmful and irreversible effects on the biological functioning and quality of the soil could result from significant accumulation of this element in surface soils (Ranjard et al. 2006). To avoid environmental risks, the permitted amounts of copper are being reduced stepwise in Switzerland and other parts of Europe. Repco (Replacement of Copper Fungicides in Organic Production of Grapevine and

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Apple in Europe, REPCO 501452) aims to contribute to the replacement of copper fungicides in organic agriculture by screening and evaluating potential agents of resistance and organically-based fungicides and developing new management measures. New products or strategies will be acceptable only if their efficacy is durable over time. *Plasmopara viticola* is included in the list of plant pathogens showing a high risk of development of resistance to fungicides (EPPO/OEPP 1999). Therefore, a method to detect the potential threat of selection for resistance to fungicides is needed for testing a large panel of products with very diverse and partially or totally unknown modes of action. This is needed even for compounds with indirect modes of action, such as plant resistance inducers, that should, at least theoretically, remain excluded from resistance risk (Gullino et al. 2000).

In this study we implemented the method of Grünwald et al. (2006) because of the strong similarities between the *Phytophthora infestans* population in the Toluca valley and *P. viticola* in Europe. Sexual reproduction, the presence of two mating types, high genetic diversity and exposure to many fungicides characterize *P. infestans* in the Toluca valley (Grünwald et al. 2006) as well as *P. viticola* populations in Europe, and should enhance the chance of detecting selection for resistance within a field season (Grünwald and Flier 2005). As done by Grünwald et al. (2006), we tested the hypotheses that exposure to fungicides would lead to (1) a shift in the sensitivity distribution (i.e., selection) and (2) a lower genotypic diversity in the population. In our study we followed the evolution of three populations throughout one season. One population was sprayed with azoxystrobin (Q-pop), a compound belonging to the QoI fungicides, reported as engendering selection pressure and resistance (Heaney et al. 2000, Zheng et al. 2000), a second population was sprayed with the organically-based fungicide Mycosan (M-pop), and a third population was not sprayed (U-pop). We followed the changes in population structure of the three populations with neutral specific SSR markers (Gobbin et al. 2003) and with the specific marker for strobilurin resistance (Chen et al. 2004), as control for resistance selection.

Samplings were performed as described by Gobbin et al. (2003) in the experimental vineyard at Fibl in Frick (Switzerland). The plot consisted of susceptible vines of Müller–Thurgau and Chasselas planted alternately. The distance between rows was 2 m; distance within the row was 1.1 m. Three subplots were designed, each composed of 16 vines planted in two rows (four vines of Müller–Thurgau and Chasselas each per row). The distance between the subplots was 1.1 m. Vines of the first subplot were not treated with fungicides (U-pop, negative control), vines of the second subplot were treated with Mycosan (50% acidified clay, 41% wettable sulphur, 1% *Equisetum* extracts, Andermatt Biocontrol, Grossdietwil; M-pop) at 0.8% dosage. Vines of the third subplot were treated with azoxystrobin (Quadris, 250 g l^{-1}, Syngenta Crop Protection, Basel, Switzerland; Q-pop) at the 0.05% dosage. The employed amount corresponded to half of the recommended rate. However, it largely exceeded the lethal dosage for *P. viticola* assessed by Genet et al. (2006) in a whole plant bioassay, where disease control of >90% was achieved with a fungicide concentration of 4 μg ml^{-1}. Plants were treated 14 times from 19 May until 10 August 2004 (19, 27 May; 02, 04, 09, 15, 21, 28 June; 05, 12, 19, 26 July; 02, 10 August). First observation of *P. viticola* was on 9 July in the U-pop. The course of the season was characterized by two dry periods (25 July–05 August and 27 August–17 September) followed by rainy periods that favoured a strong disease increase.

A total of 481 lesions (U-pop: 241, M-pop: 88, and Q-pop: 152) was collected on four sampling dates: 14 July (only in the U-pop), 27 July, 16 August and 21 September. During the first and second samplings, samples from all detectable lesions were collected, whereas by the third one, sample number per vine was limited to a maximum of nine lesions. By the last date the disease showed a mosaic pattern and only well defined lesions were sampled. Collected samples, consisting of half a sporulating lesion (about 1 cm^2, including some healthy leaf tissue) excised with a cutter, were assigned coordinates to locate their exact position in the vineyard (Gobbin et al. 2003).

DNA extraction was performed as described in Gobbin et al. (2003). Frequency of strobilurin-resistant genotypes was assessed by amplification of part of the *cyt b* gene fragment and digestion of 5 μl PCR products with 0.3 U of Fnu 4 HI (New England Biolabs) for 6 h at 37°C. Amplification was performed in a volume of 15 μl consisting of 1× reaction buffer (Pharmacia Biotechnology), 0.14 μM of both forward (COB_279F, Chen et al. 2004) and reverse primers

(StrobiR: 5′-CCACTCAGGAACAATATGTAAAGG-3′, designed on sequence AX577569, Burbidge et al. 2002), 0.20 mM of each dNTP, 0.07 U μl^{-1} Taq polymerase (Pharmacia Biotechnology) and 5 μl of DNA (not quantified). Amplifications were performed in a thermal cycler programmed for an initial denaturation of 2 min 30 s, followed by 40 cycles of amplification with the following parameters: 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by 10 min at 72°C. Digested fragments were separated on 1.2% agarose gels. The mutation conferring resistance to strobilurin was present in 56.9, 36.5 and 93.8% of the samples collected in the U, M and Q-pop, respectively. The observed shift in resistance frequency with respect to the unexposed population (U-pop) could indicate on one side a selection toward resistance (Q-pop) or on the other side a selection of susceptible wild-type genotypes (M-pop).

The selection of a resistant population and the subsequent reduction of efficacy of the applied fungicide in the Quadris-treated plot are consistent with the field observations. Indeed, severity in the Q-pop was only slightly lower than in the U-pop and at the end of September vines of the two plots were almost completely defoliated. Disease in the Mycosan-treated plot progressed more slowly and vines were slightly healthier at the end of the season. The reduced frequency of strobilurin-resistant mutants in the Mycosan plot compared to the untreated plot could be a consequence of a fitness cost in these individuals, which are more frequently eliminated when exposed to the stress induced by the partial control of Mycosan, than the

more fit wild-type strobilurin-susceptible individuals. This would be in accord with the results of Genet et al. (2006), where a recovery of sensitivity of the resistant *P. viticola* population after consecutive transfers on unsprayed plants was observed, suggesting that the resistant phenotypes are less competitive than the sensitive ones. A fitness penalty of strobilurin-resistant mutants in *P. viticola* even under optimal growth conditions was observed also by Heaney et al. (2000).

The hypothesis formulated by Grünwald et al. (2006) that genotypic diversity would decrease in a population exposed to fungicide compared with the unexposed population was tested. PCR amplification of the four *P. viticola*-specific SSR loci ISA, CES, BER and GOB and sequencer-based fragment analysis were performed according to the protocol described by Gobbin et al. (2003). The complete genotyping (at least one allele per each locus and sample) was successful for approx. 61% of the samples collected (293 out of 481). Sixty-five, 35 and 25 SSR genotypes were discriminated respectively among the 145 (U-pop), 55 (M-pop) and 93 (Q-pop) lesions genotyped at all four loci (Table 1). In all three subplots the same genotype occurred at the highest frequency; it composed 46.2% (U-pop,), 32.7% (M-pop) and 65.5% (Q-pop) of the respective populations throughout the season. The highest proportion of genotypes detected only once throughout the epidemiological season was shown by the M-pop, followed by the U-pop and Q-pop.

Multilocus genotypic diversity analyses were performed as described by Grünwald et al. (2006).

Table 1 Genotypic diversity of grapevine downy mildew (*Plasmopara viticola*) populations based on analysis of four specific SSR loci ISA, CES, BER and GOB, and % of strobilurin resistant mutants

	N^a	g^b	g55[c]	H^d	E_5^e	R^f
U-pop	145	65	26	2.87	0.2211	56.9
M-pop	55	35	35	2.98	0.4544	36.5
Q-pop	93	25	15	1.70***	0.2951	93.8

[a] Individuals genotyped at all four SSR loci

[b] Number of genotypes observed

[c] Expected number of genotypes calculated for a sample size of $n=55$ (the largest common sample size to be compared) isolates per population estimated using the rarefaction method (Grünwald et al. 2003)

[d] Shannon–Wiener diversity index (Grünwald et al. 2003; Shannon and Weaver 1949). The value followed by *** indicates that H in this population was significantly different ($P<0.001$) from the H of the U-pop according to pairwise, Bonferroni-corrected t-tests

[e] Evenness (Grünwald et al. 2003; Ludwig and Reynolds 1988)

[f] Percent of individuals carrying the mutation conferring resistance to strobilurin

The highest genotypic diversity, estimated by Shannon–Wiener's index H (Shannon and Weaver 1949), was observed for the M-pop ($H=2.98$), followed by the U-pop ($H=2.87$) and by the Q-pop ($H=1.70$) (Table 1). A t-test (Magurran 1988) with a Bonferroni correction for multiple comparisons was implemented to assess the significance of the difference between genotypic diversities within the unexposed U-pop and the fungicide-exposed Q-pop (Table 1). Evenness index E_5 (Ludwig and Reynolds 1988) was calculated and genotypic richness was estimated using rarefaction curves based on the sample size of the smallest population (M-pop: $N=55$) using the algorithm <Rarefac.c> (Grünwald et al. 2001, 2003). The Q-pop became the most clonal, had the lowest expected number of genotypes and intermediate evenness E_5 (Table 1), indicating a reduction in genetic diversity of the *P. viticola* population, consistent with directional selection toward higher resistance. The U-pop had an intermediate expected number of genotypes estimated by rarefaction and the lowest E_5 evenness. The M-pop was the least clonal and had the highest expected number of genotypes estimated by rarefaction, and the highest E_5 evenness. All analyses consistently led to the conclusion that genetic diversity is reduced in the *P. viticola* population exposed to selection pressure by strobilurin fungicides (Q-pop). Therefore, the method implemented by Grünwald et al. (2006) is effective for detecting selection in *P. viticola* populations genotyped by neutral specific SSR markers and therefore could be a valid instrument for screening a large panel of products with non-specific, different and possibly indirect modes of action. It is important to note that the fact that selection is not detected in a season does not indicate that resistance could not evolve (Grünwald et al. 2006).

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