2010-ACT-11

Multilocus genotyping of CAA fungicide resistant and susceptible grapevine downy mildew isolates infer a lack of population differentiation at both temporal and spatial scales

V. Machefer[a], S. Ahmed[a], MP. Latorse[b], R. Beffa[b], F. Delmotte[a]

[a]INRA, UMR 1065 SV, Institut des Sciences de la Vigne et du Vin, Centre Bordeaux-Aquitaine, BP 81, 33883 Villenave d'Ornon cedex, France[b] Bayer SAS, Bayer CropScience, 14 impasse Pierre Baizet, F-69263 Lyon Cedex, France

---

Plasmopara viticola (Berkl. and Curt.) Berl. and de Toni., the causal agent of grapevine downy mildew disease, is a native species from North America that is believed to have been introduced into Europe in the late 1870s. The disease then spread rapidly through most of continental Europe to become one of the most important vineyard diseases today. At present, chemical control is the most effective measure used to protect grapes from downy mildew disease.

In response to the systematic control of this disease based on fungicide treatments, the pathogen has evolved genetic resistance for several classes of fungicides. Since fungicide development is a lengthy and expensive process, increasing our knowledge as to the mode and rate of evolution of resistance is of great importance. The development of effective strategies for the control of pathogen populations therefore requires precise understanding of the conditions under which genetic resistance to fungicides appear, how this trait is transmitted and subsequently maintained in natural populations.

The class of carboxylic acid amide (CAA) fungicides includes mandipropamid, benthiavali-carb, dimethomorph, flumorph, iprovalicarb and valifenalate. Resistance to CAAs in *P. viticola* field populations has been reported in France and Germany for almost 10 years, with cross resistance shown between the representatives of this fungicide family. The mode of action of CAA has been recently described by Blum *et al.* (2010) who have demonstrated that one recessive mutation (G1105S) in PvCesA3 gene (putative cellulose synthase) causes inheritable resistance to the CAA fungicide.

In this study, using neutral nuclear markers we have investigated the genetic variability of a set of samples of *P. viticola* that have been subjected to CAA treatment. Here, we compare the microsatellite diversity of groups of samples that are either resistant or susceptible to CAA. We have performed a population genetic analysis of the data in order to investigate the existence of population partitioning in resistance and susceptible individuals at both temporal and spatial scales. We also compare the genetic diversity of samples collected from different treatment trials in order to determine whether there is any difference between *P. viticola* diversity in treated and untreated areas.

Materials and methods

Sampling design. A vineyard located at Bligny in Champagne (France) was divided into three large plots of minimum 400 m^2 corresponding to three different management strategies: the first plot was the control plot (untreated), the second plot was treated with a non CAA mixture called Mikal flash (fosetyl-aluminium and Folpel) and the third plot was treated with sirbel UD, a CAA fungicide mixture (Iprovalicarbe and Folpel). These fungicides were used with a rate of application of 4 kg/ha and 1.3 kg/ha respectively. For each year, fungicides were applied three times after the first sampling date of collection, with an interval of 14 days between each treatment. Therefore, isolates of *P. viticola* collected at the second date of each year correspond to isolates that have been treated with the respective fungicide (Mikal Flash or Sirbel UD). It is worth noting that, in this protocol, the fungicides were applied in eradicant situations.

From these three plots, a total of 923 isolates of *P. viticola* were collected from single lesions on five occasions during three consecutive years (Table 1).

Fungicide sensitivity tests. Leaf disk bioassays have been used to characterize the isolates for their sensitivity to CAA. Inoculum from each single lesion was first multiply by one biological cycle onto leaves of *Vitis vinifera* cv. Cabernet Sauvignon.

A discriminatory rate of iprovalicarb (30 mg/l) was selected and mixed with the sporangia suspension of each isolate finally adjusted at 20000 sporangia per ml. Two 10 µl droplets of inoculum and fungicide were inoculated at the lower surface of each leaf disk put on survival medium (agar + 1% kinetine) in Petri dishes and placed at 20°C in climatic chamber with the photoperiod of 12 hours. Two days after inoculation the water of each droplet was suck up and five days later the growth and sporulation of the fungus were visually assessed. The isolates were classified in sensitive phenotype when no visible symptoms could be observed and resistant phenotype when efficacy was < 100%.

Table 1: Number of *Plasmopara viticola* isolates collected in Bligny vineyard according to the three plot management strategy (Non CAA treated, CAA treated, Untreated control) and to the date of the collect. Control plot is untreated, non CAA treated plot is treated with Mikal flash (fosetyl-aluminium and Folpel) and CAA treated plot is treated with sirbel UD (Iprovalicarbe and Folpel). For the CAA treated and the non-CAA treated plots, three fungicide treatments were applied between the first and the second sampling dates of collection of each year.

	07/06/2006 pre-treatment	10/07/2006 post-treatment	12/06/2007 pre-treatment	02/08/2007 post-treatment	01/07/2008 pre-treatment	Total
Non CAA treated	33	28	77	65	93	296
CAA treated	32	31	69	81	98	311
Untreated control	23	30	83	77	103	316

Multilocus genotyping. A subset of 402 isolates was selected in order to investigate the partitioning of resistant and sensitive *P. viticola* populations (Table 2). Isolates were extracted from infected plant tissue as described by Delmotte *et al.* (2006) and genotyped using eight polymorphic microsatellite markers (Delmotte *et al.*, 2006; Gobbin *et al.*, 2003) and three single nucleotide polymorphism derived from an expressed sequence tag library provided by Bayer CropSciences (Table 3). Microsatellites analysis was automated on a Beckman Coulter Ceq 8000 capillary sequencer and SNPs were transformed into cleaved amplified polymorphism sequence (CAPS) markers.

Statistical analyses. The numbers of resistant and sensitive isolates and their spatial distribution in their respective plot was plotted for each date (Figure 2) and a pairwise chi squared tests were performed to compare plots at each sampling date. Departures from Hardy-Weinberg expected frequencies, observed and expected heterozygosity, allelic richness, pairwise *F*st (Weir and Cockerham, 1984) and pairwise linkage disequilibrium were measured in Genepop v4 (Raymond et Rousset, 1995). The presence of repeated multilocus genotypes was detected using Genclone 2.0 (Arnaud –Haond and Belkir, 2007).

A hierarchical analysis of molecular variance (AMOVA) was performed in ARLEQUIN version 3.11 (Schneider *et al.*, 2000) in order to measure the level of genetic differentiation between fungicide resistant and susceptible individuals and also between temporal sampling efforts within the two respective groups. This was then repeated for different plots within the two main group

Table 2: Number of *Plasmopara viticola* isolates selected for the population genetic structure analysis according to their susceptibility to CAA fungicide (R= resistant to CAA, S=sensitive to CAA).

	07/06/2006		10/07/2006		12/06/2007		02/08/2007		01/07/2008		Total
	R	S	R	S	R	S	R	S	R	S	
Non CAA treated	6	23	8	18	8	15	6	3	23	1	111
CAA treated	11	21	28	2	16	23	15	8	27	10	161
Untreated control	5	13	11	16	13	18	11	11	25	7	130

Table 3: Characteristics of the three SNP (Single Nucleotid Polymorphism) that were transformed into CAPS (Cleaved Amplified Polymorphism Sequence) markers.

Locus Name	Homology	Primers sequence (5'-3')	Size (pb)	Restriction enzyme	Allele 1		Allele 2	
					Identity	Size (bp)	Identity	Size (bp)
Pvi1	Hypothetical protein	L: CCGTGACTCCCTTGTATTCC R: AACGAATAGGGTGCGTAGGA	494	*Pvu* I	C	302/192	T	494
Pvi12	Ubiquitin	L: CTGACGGGCAAGACCATTAC R: GAACACACCAGCACCACACT	372	EciI	G	209/163	A	372
Pvi13	Peptidyl-prolyl isomerase	L: CCAAGTCGCAAGCAAGTAAA R: GCGAAAAAGGAAAAATAAGCA	638	HgaI	C	472/211	A	638

Results

The incidence of grapevine downy mildew infections in Bligny was relatively low in the year 2006 which enabled the exhaustive sampling of isolates, but high in 2007 and 2008. The number of resistant isolates sampled overall in the field increased over the three years of the study (Figure 1). However, this overall general trend masks the variation that exists in the number of resistant isolates found among plots for temporal sampling events. The proportion of resistant isolates collected in June 2006 (before treatment) was low and we detected no significant difference between each plot (P=0.18). Collections from July 2006 (after treatment) showed a higher proportion resistant isolates in the CAA plot compared to non CAA treated plot and untreated control plot. In June 2007 (before treatment and early in the season) samples revealed a decrease in the number of resistant isolates compared to the previous date but with no significant difference detected between plots. In August 2007, the samples collected 6 weeks after the treatments showed a higher proportion of resistance in the CAA treated plot than in the non CAA treated plot. In July 2008 (before treatment but late in the season), the mean proportion of resistant isolates overall reached 80%, with a slightly higher percentage being found in the non CAA treated plot compared to the CAA and untreated plots. For all the three years for which data were collected, we found no spatial aggregation of resistant or susceptible *P. viticola* isolates in the field.

The genotypic data were then grouped into two populations according to whether they were susceptible or resistant to CAA (called S or R respectively). Based on the genotypes derived from 11 nuclear markers, we identified 166 different multilocus genotypes among the 213 resistant isolates (G/N=0.78) and 173 among 189 sensitive isolates (G/N=0.91). Multicopy genotypes were represented no more than two or three times, except in 2008 where one resistant genotype was found in 17 other isolates. The level of genetic diversity estimated for R and S populations at each temporal sampling event showed no significant deviations from Hardy-Weinberg expectations. The number of significant tests for linkage disequilibrium detected was also low implying a largely sexual breeding system for both resistant and sensitive populations (Table 4).

Pairwise F_{ST} estimates between resistant and sensitive populations were low and not significant (excepted for July 2008) revealing little or no differentiation between the resistant and susceptible populations. Clustering analysis in the program Structure also suggests a single population.

For the hierarchical analysis, when isolates were grouped according to their susceptibility to CAA and subpopulations formed according to temporal sampling events, we found that only 0.3% of the total genetic variance could be attributed to divergence between the two main groups and 2.4% to differentiation within groups but among temporal population (Table 5). The same analysis performed with two groups (R/S) and populations represented by the treatment plots, revealed that 0.85% and 0.19% of the genetic variance resulted from group and population effect respectively, suggesting that the observed diversity can be explained at the individual level rather than between resistant or susceptible groups, or temporal and treatment plot subpopulations.

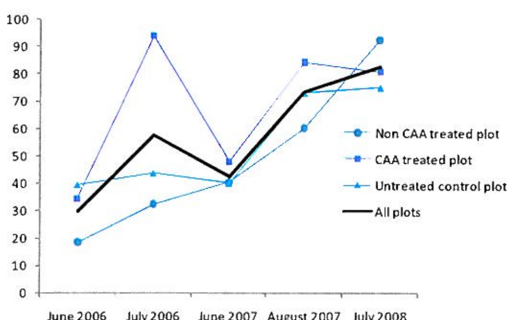

Figure 1: Time course evolution of the frequency of resistant *P. viticola* isolates within Bligny field according to the plot management strategy.

Discussion

Many interacting factors are involved in the emergence and spread of alleles that confer resistance to fungicide treatment in pathogen populations. The evolution of fungicide resistant alleles is dependent on the mode of action of the fungicide, the selection pressure on the pathogen response to the fungicide, the abiotic conditions for disease development, and the potential for evolving genetic resistance in the pathogen (such as breeding system, recombination rate, mutation rate, dispersal capability).

In this study, we have shown that the number of resistant isolates sampled increased globally over the three years in all plots (Figure 1). To allow the collect of representative number of single lesions at the beginning of the season, fungicide applications were used in eradicant situations (i.e. when disease epidemics is already in its exponential growth), a situation that did not fit classical agronomical practises. This result reinforces the recommendation to avoid applying CAA fungicides after downy mildew infections have started, especially in situations where resistance to the fungicide is suspected. It is also possible that, in 2008, a year that presented favourable conditions to the initiation of downy mildew epidemics, dispersion from surrounding vineyards where CAA fungicides have been intensively used may have contributed to increase the frequency of R isolates in the Bligny vineyard.

When one considers each temporal group in greater detail, the number of resistant isolates sampled was significantly higher in the CAA plot only after the treatment in 2006 (Year 1). Conversely, in 2008 (Year 3) before treatment in the target plot but after treatment in the surrounding vineyards, we found that the number of resistant isolates was greater in the non CAA treated plot compared with the other two. In terms of spatial genetic structure we found that CAA treatment does not affect the spatial distribution of resistant isolates (Figure 1) and no spatial aggregation was observed. These results are congruent with dispersal of *P. viticola* at the meters scale leading to population mixing across the plots.

The genetic diversity we observe in *P. viticola* could almost entirely be attributed to individual differences rather than temporal or plot differences being they resistant or susceptible to CAA fungicide.

Table 4: Comparison of genetic characteristics of resistant vs. sensitive populations of *P. viticola*.

	R						S					
	07/06/2006	10/07/2006	12/06/2007	02/08/2007	01/07/2008	Total	07/06/2006	10/07/2006	12/06/2007	02/08/2007	01/07/2008	Total
N	22	47	37	32	75	213	57	36	56	22	18	189
MLG	19	42	36	32	47	166	55	36	53	21	17	173
G/N	0.86	0.89	0.97	1	0.63	0.78	0.96	1	0.94	0.95	0.94	0.91
S	17	38	35	32	40	145	53	36	50	20	16	14
Max	3	3	2	-	17	17	2	-	2	2	2	4
Allelic richness	2.09	2.36	2.36	2.45	2.27	2.54	2.36	2.45	2.64	2.18	2.36	2.90
He	0.28	0.31	0.32	0.33	0.33	0.33	0.31	0.32	0.33	0.34	0.31	0.33
Ho	0.37	0.36	0.35	0.34	0.40	0.37	0.38	0.37	0.34	0.35	0.33	0.36
$P_{deficit}$	0.99	0.97	0.94	0.3	1	1	0.83	0.7	0.01	0.6	0.29	0
P_{excess}	0.02	0.03	0.06	0.7	0	0	0.17	0.32	0.99	0.41	0.7	1
LD	0/55	3/55	2/55	1/55	23/55	23/55	2/55	2/55	5/55	2/55	3/55	4/55

N: number of individual, MLG: multilocus genotype, G/N: number of multilocus genotype/total number of individuals, S: number of singleton, Max: maximum number of repetition on a MLG, Allelic richness: average number of allele, He/Ho : expected and observed heterozygositie, *P* : excess and deficit heterozygositie, LD : linkage disequilibrium.

Table 5: Hierarchical analysis of molecular variance for samples of *Plasmopara viticola* grouped by their susceptibility or resistance to CAA (groups) and by date or by plots (populations).

Source of variation	Degrees of freedom	Variance components	Percent of variation	Fixation indices
Among groups (R/S)	1	0.006	0.34	F_{CT}=0.00336 *
Among dates within groups	8	0.045	2.48	F_{SC}=0.02486 *
Within populations	794	1.781	97.2	F_{ST}=0.02813 NS
Among groups (R/S)	1	8.555	0.85	F_{CT}=0.00854 NS
Among plots within groups	4	0.003	0.19	F_{SC}=0.00188 *
Within populations	798	1.813	99,0	F_{ST}=0.01040 NS

F_{CT}: estimated fixation index among differnet groups; F_{SC}: estimated fixation index within a region; F_{ST} estimated global fixation index within populations
* P<0.01, NS: not significant

With regard to breeding system, we know that this pathogen has the capacity for both sexual and asexual reproduction. Analysis of the genotype data revealed no significant deviations from Hardy Weinberg expected frequencies and linkage disequilibrium was low. In addition, we found a low number of multiple matching multilocus genotypes, inferring a low incidence or absence of clonal reproduction except at year 3 after treatment (2008) (Table 4). Together these observations would suggest that the breeding system in ours plots is random and predominantly sexual between resistant and susceptible isolates. These results also confirm the importance of primary inoculum in the epidemics of grapevine downy mildew.

In conclusion, this study suggests a lack of both spatial and temporal genetic structure between R and S populations of *P. viticola*. No genetic structure was detected in the set of genotyped samples, suggesting a single panmictic population where all isolates have the potential to breed with each other regardless of fungicide susceptibility and their plot origin. Therefore, in the absence of fungicide selection pressure, the wild type (S) and the mutated (R) alleles are fully reshuffled into *P. viticola* genotypes by the sexual reproduction. Since the resistance to CAA has been described as recessive (Blum *et al.* 2010), an annual generation of sexual reproduction results in a decrease of resistant isolates (RR) due to the formation of heterozygotes genotypes (RS) that are sensitive. Therefore, rotation with other fungicide compounds having other modes of action is providing a good strategy to manage resistance to this class of fungicide.

Literature cited

Arnaud-Haond S, Belkhir K. 2007. Genclone: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Mol. Ecol. Notes* 7: 15–17.

Blum M, Waldner M, Gisi U. 2010. A single point mutation in the novel PvCesA3 gene confers resistance to the carboxylic acid amide fungicide mandipropamid in *Plasmopara viticola*, *Fungal Genetics and Biology*, in press.

Delmotte F, WJ Chen, Richard-Cervera S, Greif C, Papura D, Giresse X, Mondor-Genson G, Corio-Costet MF. 2006. Microsatellite DNA markers for *Plasmopara viticola*, the causal agent of downy mildew of grapes. *Mol. Ecol. Notes* 6: 379–381.

Gobbin D, Pertot I, Gessler C. 2003. Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR analysis. *European Journal of Plant Pathology* 109: 153–164.

Raymond M, Rousset F. 1995. GENEPOP (v.1.2): population genetic software for exact test and ecumenism. J. Hered. 86: 248–249.

Schneider S, Roessli D, Excoffier L. 2000. Arlequin ver 2.000. A Software for Population Genetics Analysis. Genetic and Biometry Laboratory, University of Geneva, Switzerland.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.

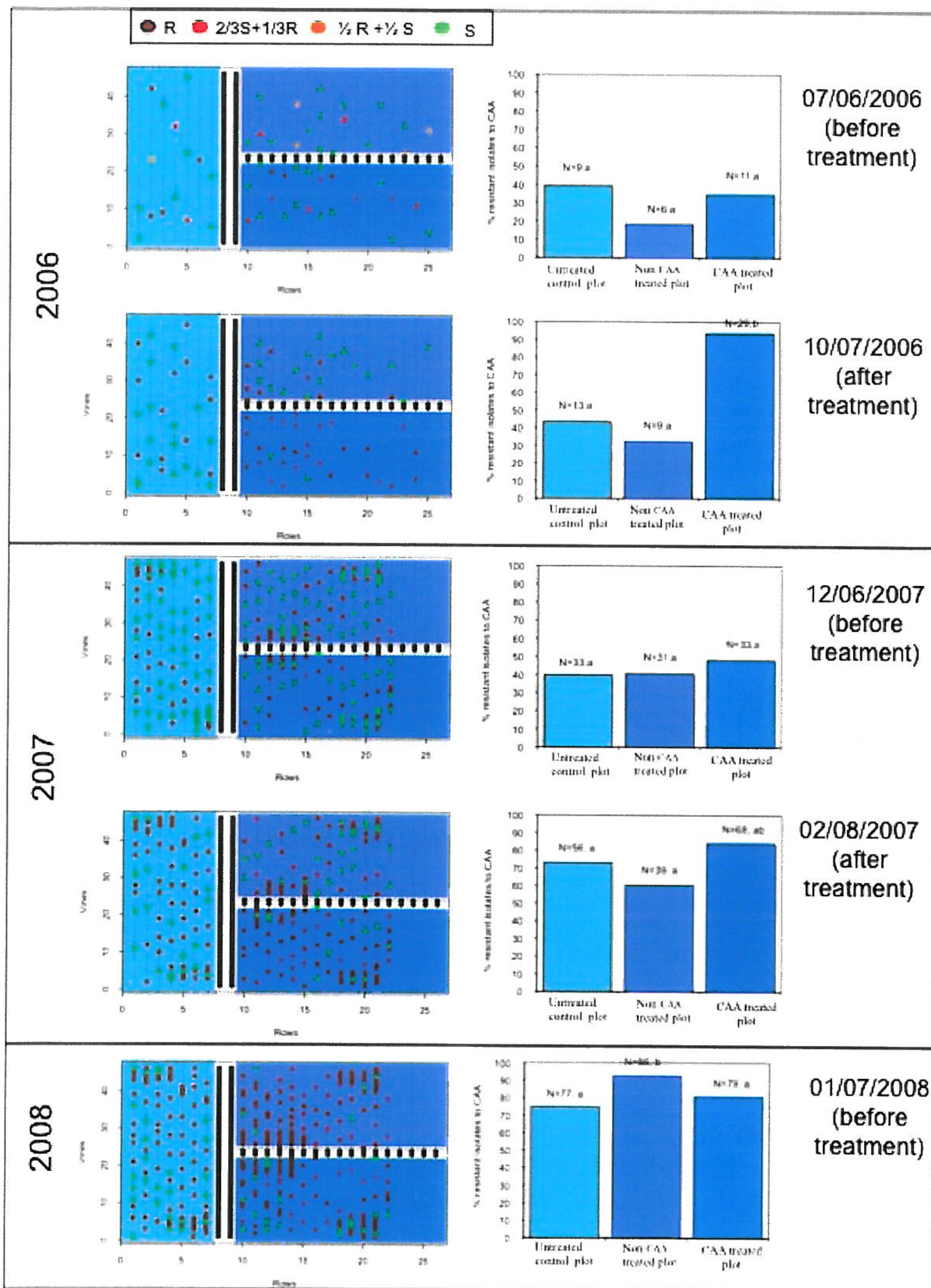

Figure 2: Spatial representation of the *P. viticola* isolates collected within the vineyard at the five dates (green=sensitive; red=resistant). The graph represents the percentage of resistant to the CAA fungicide for each date, depending on the 3 treatment modalities. The date 01/07/2008 is before treatment on the target plot (but after treatment in the surrounding vineyards).