

Training Period Report
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Downy Mildew Adaptation to Fungicide Pressure: Fitness Study

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Introduction

Downy mildew is a fungal-like disease that is specific to grapevine and can attack all tissues with stomatas. Symptoms are yellow "oilspots" or white down spots on leaves, brown areas on shoots, and fruits rot. The causal agent is *Plasmopara viticola*, a biotrophic parasite in the family of Peronosporaceae (oomycota) that can only develop on its host and doesn't survive without grapevine.

That disease was brought from United States and first occurred in 1878 in France. Today, it is still responsible for extensive damage in all French vineyards. At present, chemical control remains the most effective form of management, and this parasite is focusing 78% of all fungicides applications in France. Many efficient molecules have been developed, but resistant pathogen populations emerged regularly and rather quickly, one example being general resistance reported to QoI fungicides, that inhibitates mitochondrial respiration by stopping the electron transport chain. In this study we focuse on *Plasmopara viticola*'s resistance to famoxadone, which has been demonstrated to be due to a punctual mutation (G143A) in the mitochondrial gene coding the cytochrom *b*.

A major issue understanding the resistance process is to measure pathogen fitness, i.e. the selective value of the pathogen. The study's general goal is to acquire datas on dispersion and maintain of *P. viticola* resistant strains, in the optics of improving fungicides applications management. My training period specific goal focuses on the study of fitness and competitiveness of famoxadone resistant and sensitive strains, in order to answer the following question :

Is there a cost of resistance, i.e. are resistant strains less competitive than sensitive ones in the absence of fungicide ?

Detecting a cost of resistance in *Plasmopara viticola* could lead to interesting repercussions in the way fungicides are used in wineryard.

Previous datas on resistant and sensitive strains fitness were collected by measuring different parameters (growth rate, sporulation rate, infectiousness, ...), and didn't proved any clear global differences between strains. In this study, we set up a competitiveness test between resistant and sensitive, in order to detect or not a cost of resistance. The experiments can be separated in three chronological points :

1. From initial mixes of resistant and sensitive strains, we will mesure during eight weeks the evolution of each proportions.
2. Molecular tools will be used to corroborate and improve the biological measures : Quantitative PCR will be performed in order to detect or not the G143A mutation.
3. An evolutionnary model will be realized with fitness datas.

1 Material and Methods

1.1 Competitiveness test

Characteristics and FIC¹ of *Plasmopara viticola* isolates resistant and sensitive couples used are detailed in table 1.

Strain	Origin	Year	FIC	QoI Resistance
COU15	Petit Couhins (33)	2003	0,648	S
MIC128	Castillon (32)	2005	0,636	R
PIC59	Couhins (33)	2003	0,571	R
FEM03	Puligny-Montrachet (21)	2003	0,731	S
PAU32	Pauillac (33)	2003	0,389	R
COU15	Petit Couhins (33)	2003	0,648	S
PAU32	Pauillac (33)	2003	0,389	R
MIC103	Fleurie (69)	2004	0,465	S

TAB. 1 – Origin and FIC of resistant and sensitive strains used in the competitiveness test

General protocol of downy mildew pricking out : Fungus isolates are cultivated on detached leaves placed on water-saturated filter paper in Petri dishes at 21°C. All experiments are performed with detached leaves of 4 weeks-old plants of Cabernet-Sauvignon grown in greenhouses.

Sporangias are taken from an infected leaf with a brush to realize a suspension in permuted water, adjusted to 40000 sporangias.mL⁻¹ with the help of a Malassez cell. 24 drops of 15μL are deposited per leaf. After one night at 22°C to allow stomatas opening and leaf infection, drops are dried with an air pump and dishes are conserved during one week minimum at 21°C.

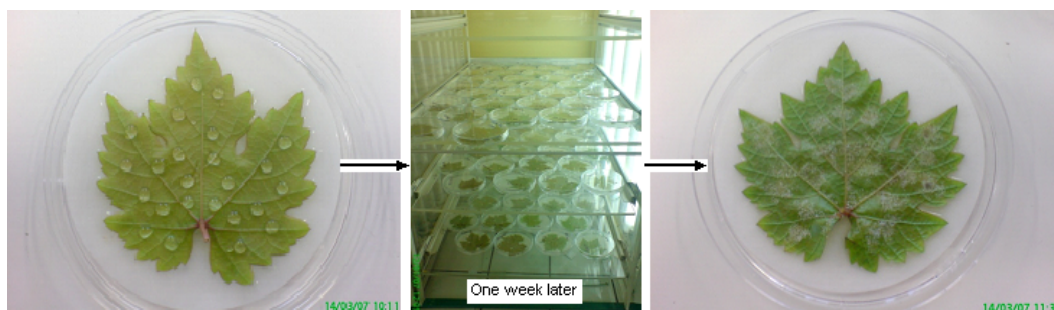


FIG. 1 – Downy mildew growth on a leaf (*in vitro* experiment)

¹FIC = Fitness Index Complex = sporulation capacity × infection frequency × latency period × sporangia size × infection efficiency × growth rate

Competitiveness test protocol : For each resistant/sensitive couple (see Table 1), three initial proportions (20% resistant, 50% resistant and 80% resistant) are realized at 40000 sporangias/mL and inoculated on four independant leaves for each proportion and each couple. After one week of growth, the mixes are pricked out on four new leaves. The experiment is repeated during eight weeks. Every week, mixes samples are taken to perform a fungicide crible and a quantitative-PCR mesure to estimate the new resistant/sensitive proportions (see below).

The entire experiment is repeated independently twice a week.

1.2 Fungicide crible

In the same time leaves are inoculated with the resistant/sensitive inoculum suspension for the competitiveness test, this suspension is used to inoculate leaves disks containing fungicide. For each proportion and each couple, two Petri dishes containing eight disks are prepared. Disks are sprayed with famoxadone at 100mg/mL, and are dried after one night of fungicide penetration. Three inoculum drops of 15 μ L are deposited on each disk.

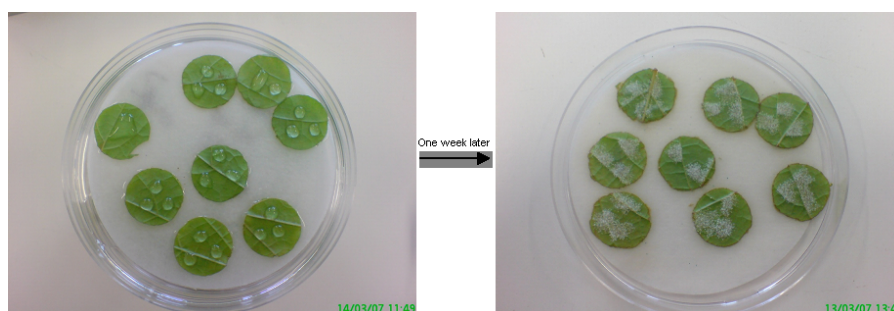


FIG. 2 – The fungicide crible

After one week of growth, stains corresponding to resistant strain remaining are observed and evaluated using a visual ladder (see figure 3, leading to estimate the initial resistant proportion that was contained in the suspension deposited on the disks).

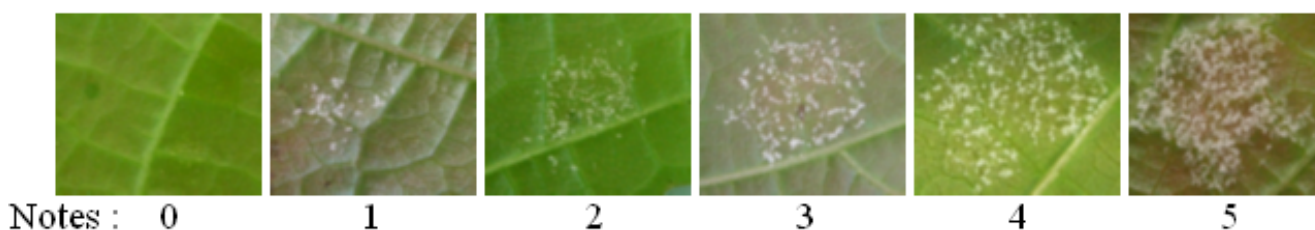


FIG. 3 – Visual ladder used in the fungicide crible

1.3 Quantitative Polymerase Chain Reaction (Q-PCR)

1.3.1 DNA extraction

For each proportion of each couple, two tubes of 0,5mL of sporangias suspension and four little leaf disks containing three mildew stains are prepared from the four leaves every week, and conserved at -20°C. DNA extraction is performed from these samples :

- Samples are centrifugated and the pellet is iced at -80°C and lyophilised overnight
- After crushing the dried pellet, 600µL of CTAB² (cetyl trimethylammonium bromide) at 65°C are added
- After one hour waiting, 400µL of isoamylalcohol-chloroform are added, and tubes are slowly shaken during ten minutes. Then, a centrifugation is performed at 13000rpm and 4°C during ten minutes, and the aqueous phase is conserved.
- 2/3 volume of cold isopropanol are added, and after shaking a two-hours wait is necessary to let the DNA precipitate.
- Tubes are centrifugated ten minutes at 13000rpm and 4°C, the pellet is conserved and rinsed with ethanol 70°, and centrifugated again. Then the pellet is dried, dissolved in 50µL of water and conserved at -20°C.

1.3.2 Q-PCR

In order to measure the resistant/sensitive proportions, a strategy based on the detection of the G143A mutation (that confers resistance) has been set up. Two couples of primers are used :

- A first couple that is unspecific to resistant or sensitive isolates, and leads to the amplification of the mitochondrial *b* cytochrom gene.
- A second couple also leading to the amplification of the cytochrom *b* gene, but in which one primer hybridates directly with the G143A mutation, and consequently is specific to resistant isolates.

Quantitative PCR is performed with these two couples of primers, and the ratio between resistant-specific primers and unspecific primers gives us the resistant isolate proportion in the mix.

The following protocol is used : 5µL of Master Mix SybrGreen³, 0,1µL of each primer and 2,5µL of available DNA completed to 10µL with H₂O are submitted to 30 cycles of quantitative PCR.

²25mL of Tris 2M pH₈, 20mL of EDTA 0,5M pH₈, 41g of solid NaCl, 10g of CTAB, and 5g of PVP-40 completed to 1L with H₂O milliQ

³SybrGreen is a fluorophore that binds to double-strand DNA, it is used to quantify the PCR amplification during the cycles

2 Results

2.1 Biological competitiveness test

2.1.1 Measures standardization

In order to determine the resistant/sensitive strains proportions in the course of time, we have to assess whether the visual notation of the fungicide crib is correct. A standard scale is realized by inoculating known proportions on leaf disks (0 - 10 - ... - 100% of resistant) under fungicide application, and after one week of growth the stains are notated between 0 and 5 using the visual ladder. Results are shown in figure 4.

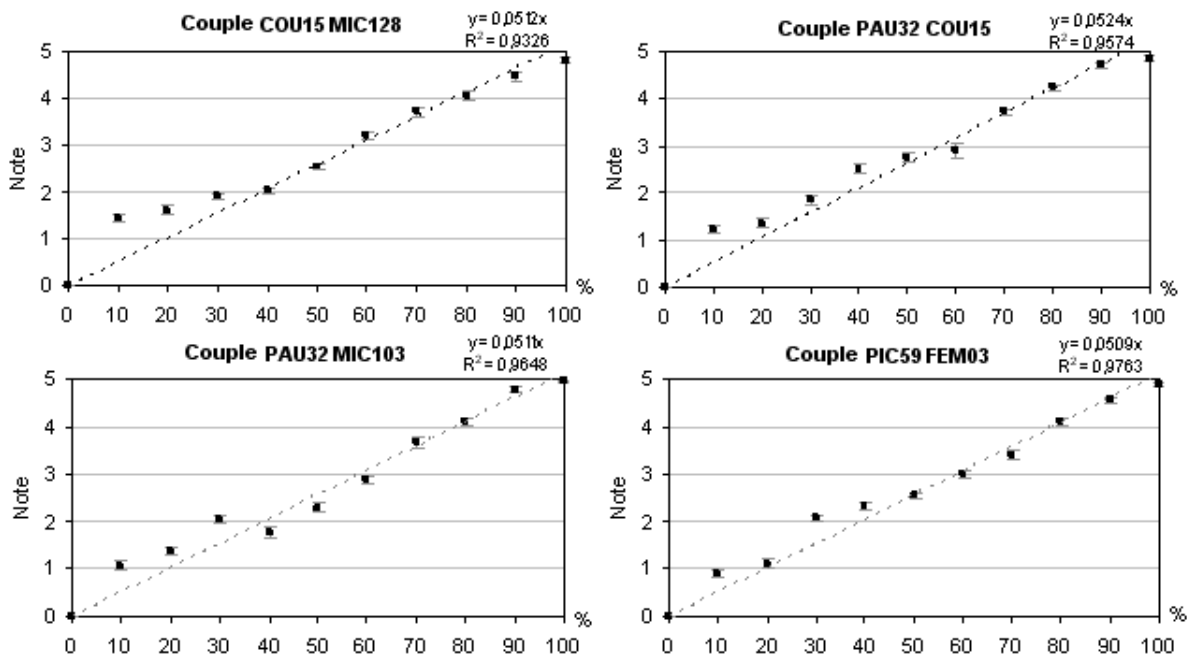


FIG. 4 – Standard scales for the four couples (error : Standard Error; dotted line : linear regression)

As we can see on those graphs (fig 4), there is a good correlation between the percentage of resistant strains and the visual notation, under the fungicide crib. Indeed, inoculating at 40000 sporangias/mL extends the visual notation from 0 to 5 in a linear way. That concentration gives us the better precision for a visual notation, and is conserved for the following experiments.

2.1.2 Competitiveness test (biological results)

Evolution of resistant/sensitive proportions is determined during eight weeks, using the fungicide crib. Two independent experiments are realized. Results of the first experiment are shown in figure 5.

At the time, a statistical study of those graphs hasn't been realized yet. For instance, a statistical work on the slopes point by point is planned. So the analyse of those graphs

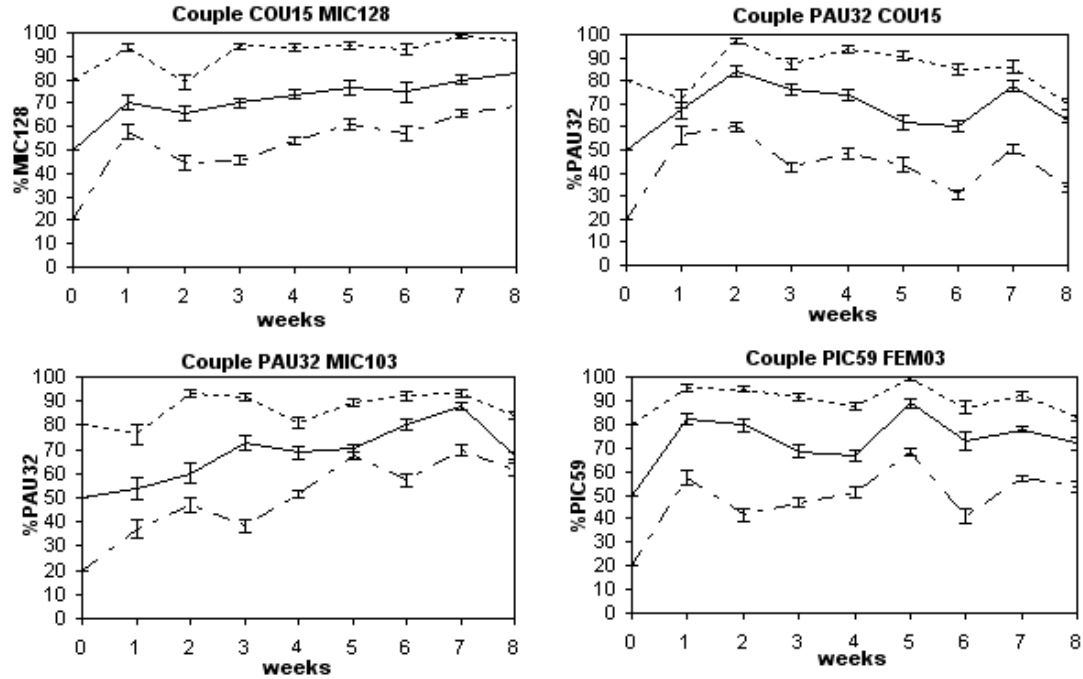


FIG. 5 – Evolution of resistant/sensitive proportions during eight weeks, starting from three known proportions (20%, 50%, 80%) [First Test].

cannot be deeply done yet, we can only observe general tendencies. In figure 5, the evolution of proportions is comparable between the different couples and the different initial proportions : we can't detect any significant variation during eight weeks. The little variations observed may be due to aleatory variation of mildew growth, and can't be interpreted as a genuine variation of the resistant/sensitive proportion.

Results of the second experiment are shown in figure 6.

In this second test, we are able to see significant variations of the evolution of the proportions. For the couple COU15 MIC128, the evolution is similar to the one in the first experiment. But for the remaining couples PAU32 COU15, PAU32 MIC103 and PIC59 FEM03, we observe an extinction of the resistant isolates PAU32 and PIC59 after eight weeks.

These results are consistent with the available datas on fitness (see table 1) : PAU32 and PIC59 have a low fitness compared to COU15, MIC103 and FEM03. According to these results, it appears that they may also be less competitive than the sensitive isolates they are associated with.

2.2 Molecular analysis of the competitiveness test

At the time, the quantification of the evolution of the proportions using the quantitative PCR method is working, but hasn't been performed on all the samples yet. Meanwhile, The first available results (datas not shown) seem to corroborate the biological mesures using the fungicide crible.

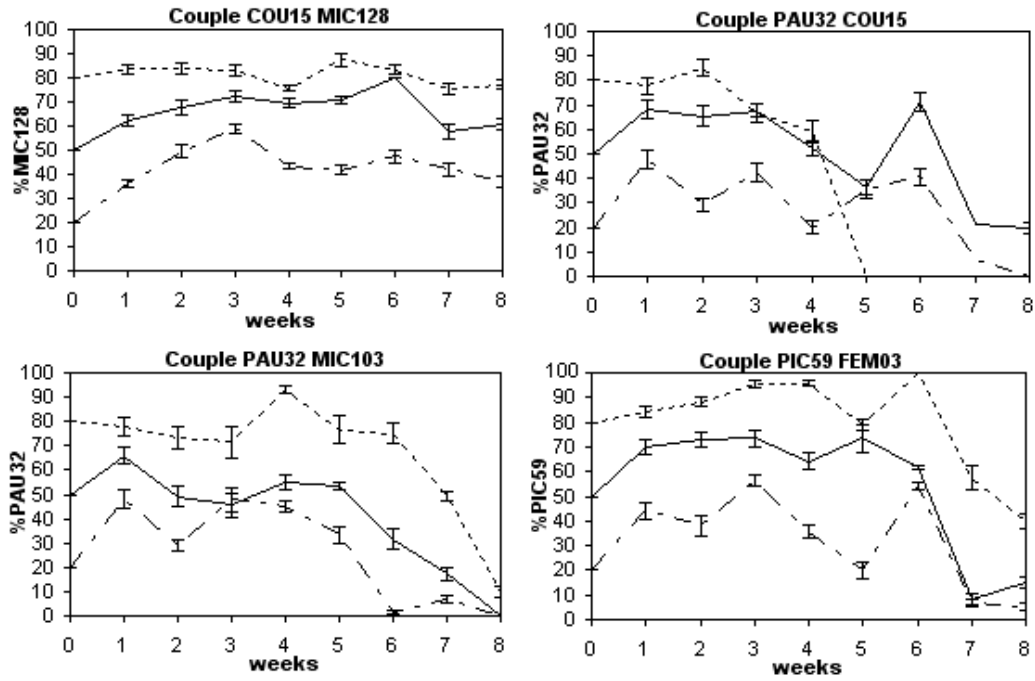


FIG. 6 – Evolution of resistant/sensitive proportions during eight weeks, starting from three known proportions (20%, 50%, 80%) [Second Test].

2.3 Modelisation

A model of evolution⁴ of a resistant/sensitive isolates mix with or without fungicide application is being realized using the fitness datas previously measured in the laboratory, to see if the experiment results are consistent or not with a theoretical modelisation.

⁴realised with Stella[®] software