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IDENTIFICATION OF A MUTATION IN THE *CYTB* GENE ASSOCIATED TO RESISTANCE TO CYAZOFAMID IN GRAPE DOWNY MILDEW

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ABSTRACT

Plasmopara viticola populations are yearly tested in bioassays for their sensitivity to cyazofamid, a QiI (quinone inside inhibitor) fungicide. These monitoring efforts allowed to detect populations resistant to cyazofamid even in the presence of SHAM (an inhibitor of the AOX alternative respiratory mechanism). The sequence analysis of the *cytb* gene of resistant isolates revealed the presence of the insert E203-DE-V204.

The agronomic impact of the presence of these resistant strains has been studied in field conditions. So far, downy mildew is still satisfactorily controlled by MILDICUT (cyazofamid 112.5 g/ha + disodium phosphonate 1125 g/ha), even in vineyards where resistant strains were detected.

This discovery of the molecular mechanism of resistance opens perspectives to improve detection and quantification methods for the monitoring of resistant populations to the QiI fungicides group.

Keywords: cyazofamid – QiI – resistance – mutation – cytochrome *b*.

RÉSUMÉ

IDENTIFICATION D'UNE MUTATION DANS LE GENE *CYTB* ASSOCIEE A LA RESISTANCE A LA CYAZOFAMIDE CHEZ LE MILDIOU DE LA VIGNE

Des populations de *Plasmopara viticola* sont testées chaque année, au moyen de tests biologiques, pour évaluer leur sensibilité à la cyazofamide, un fongicide du groupe des QiI. Cette surveillance a permis de détecter des populations résistantes à la cyazofamide, et ce même en présence de SHAM (un inhibiteur de la voie de respiration alternative AOX). L'analyse de la séquence du gène *cytb* des isolats résistants a révélé la présence de l'insertion E203-DE-V204.

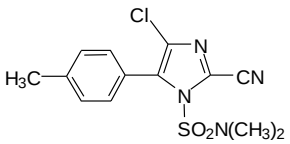
Actuellement, dans les conditions du vignoble, les essais montrent que l'infestation de mildiou reste contrôlée de façon satisfaisante par la spécialité MILDICUT (cyazofamide 112,5 g/ha + phosphate disodique 1125 g/ha), y compris lorsque la présence de souches résistantes est avérée.

Cette découverte du déterminisme moléculaire de la résistance ouvre des perspectives pour améliorer les méthodes de détection et de quantification pour le suivi des populations présentant une résistance aux fongicides QiI.

Mots-clés : cyazofamide – QiI – résistance – mutation – cytochrome *b*.

INTRODUCTION

Cyazofamid is an active substance discovered in the 1990s by Ishihara Sangyo Kaisha Ltd (ISK). Cyazofamid is a QiI (quinone inside inhibitors) fungicide belonging to the family of cyano-imidazoles in the FRAC code 21 (FRAC, 2018), effective to control Oomycete fungi by respiratory inhibition. Indeed, this active substance interferes with the mitochondrial respiration mechanism by binding to the Qi site of the cytochrome *bc*1 complex (Mitani *et al*., 2001). Cyazofamid was approved for use on grapevine in the European market in 2003.

Chemical name:	**Cyazofamid** (4-chloro-2-cyano-*N*,*N*-dimethyl-5-(4-methylphenyl)-1H-imidazole-1-sulfonamide)
Function:	Fungicide
Chemical group:	cyano-imidazole
CAS-No.:	120116-88-3
Molecular formula:	$C_{13}H_{13}ClN_4O_2S$
Molecular mass	324.8
Structure:	
Mode of action:	Complex III: cytochrome *bc*1 (ubiquinone reductase) at Qi site (FRAC target site code: C4)

MILDICUT (cyazofamid 25 g/L + disodium phosphonate 250 g/L, coded IBE 3887) is the formulation especially designed to control downy mildew in grapevine. Disodium phosphonate acts essentially by inducing the plants own defense system against attacks from fungi. It has been authorised in France since 2009; it is distributed by Belchim Crop Protection.

Grape downy mildew (*Plasmopara viticola*) is a major disease of grapevines in Europe and in France; furthermore, it is accepted as showing a high risk of development of resistance to fungicides by FRAC (2014). Therefore, an important monitoring of the potential development of resistances to cyazofamid in grape downy mildew has been running yearly since the introduction of the formulation on the market, at the facilities of Staphyt Laboratory & Greenhouse.

By comparing yearly results with the baseline, these monitoring efforts allowed first to detect cases of non-specific resistance by activation of the alternative oxidase (AOX) respiratory pathway; this aspecific resistance mechanism can be inhibited in the laboratory by the addition of SHAM (salicylhydroxamic acid). Later, shifts in sensitivity to cyazofamid were detected in 2015 and the first confirmed cases of specific resistance in 2016. The concerned strains had then to be further studied for confirmation of the resistance, complete characterisation, explanation and evaluation of the practical consequences.

In 2017, the active substance ametoctradin was added in the sensitivity tests. The mode of action of ametoctradin was recently elucidated (Deinert *et al*., 2018) as a dual inhibitor (QioI) binding to the Qo and Qi sites, with a better affinity to the Qi site. Previously, ametoctradin was referred to as a QoSI fungicide. This elucidation enables to confirm the absence of cross-resistance with cyazofamid observed in the bioassays conducted by Staphyt L&G, given that both active ingredients have distinct modes of action.

In this paper, we are focusing on 3 major aspects of our work:

1) Monitoring of the *P. viticola* populations' sensitivity to cyazofamid by ISK and Belchim since 2010;
2) Identification of a mutation in the *cytb* gene associated to the resistance to cyazofamid;
3) Practical consequences in the conditions of the French vineyard.

MATERIALS AND METHODS

MONITORING: SAMPLING AND FUNGAL MATERIAL

Since the year of introduction of the specialty IBE 3887 on the market (2010), a regular monitoring has been conducted yearly in France.

From 2010 to 2017, ISK and Belchim have collected 583 *P. viticola* population samples in 7 vineyard regions of France and had them analysed with the collaboration of Staphyt L&G. Out of this dataset, 547 representative samples were used to elaborate the present communication; the others were discarded as not relevant (specifically targeted samples for other purposes, out of the scope of the regular monitoring). As the monitoring was not done randomly, it was not designed to give quantitative indications on the presence of resistant strains in the different regions. Indeed, the objective of this monitoring was to detect the first cases of resistance by sampling in the most 'at risk' vineyards.

Last monitorings allowed to detect grape downy mildew populations characterized by different sensitivity profiles to cyazofamid. Single-sporangiophore *P. viticola* strains were isolated from these populations and these strains were then increased with preliminary subculture on untreated leaves in order to serve as inoculum for subsequent tests.

MONITORING: LEAF-DISK ASSAY

The method selected to assess the sensitivity of *P. viticola* strains to cyazofamid is leaf-disk bioassay that involves inoculating vine leaf-disks with fungicide-sporocystes mixtures.

Leaf disks preparation. Leaf disks were prepared from 'Cabernet Sauvignon' grapevine plants, produced in greenhouse (20-35°C). Leaves were selected and collected. 10 leaf disks were then cut using a cork borer and were placed with their lower surface exposed on moist filter paper into Petri dishes. Each experimental unit consisted of one Petri dish containing 10 leaf disks.

Fungicide-sporocystes mixes preparation. Aqueous solutions of cyazofamid (and ametoctradin, in 2017) were prepared. The final test concentrations were 0-1-10 mg/L of active substance ± SHAM (100 mg/L). Under fungicide pressure when the main route of mitochondrial respiration is blocked, some fungi activate an alternative respiratory pathway that is sustained by the AOX enzyme (Wood & Hollomon, 2003). In this rescue mechanism, mitochondrial electron transfer is diverted by circumventing the complex III and IV. Consequently during *in vitro* tests, the effects of fungicide inhibiting mitochondrial respiratory chain at complex III can be partially masked by activation of the AOX pathway. To avoid that, SHAM can be used as an inhibitor of the AOX which blocks the deviated flow of electrons. Therefore, if a fungus develops sporulations on the leaf-disk, even in the presence of a cyazofamid and SHAM mixture, then it would be resistant to cyazofamid. For each *P. viticola* strain, fresh sporangia were harvested with a paint brush and suspended in fresh water. The concentration of inoculum was adjusted to 10^5 sporangia per mL. Subsequently, equal volumes of fungicide solutions and sporangia suspension were mixed in order to obtain inoculum with wished fungicide and sporocystes concentration (1-10 mg/L cyazofamid or 1 mg/L ametoctradin ± SHAM and 50 000 spores/mL respectively).

Inoculation. The leaf-disks were then inoculated by deposit of droplets of fungicide-sporocystes mixture on the lower surface of each leaf-disk. In control conditions, leaf-disks were inoculated by droplets of sporocystes without fungicide. Seven days after inoculation, after incubation at 21°C, the development of downy mildew on leaf disks was determined by assessing the proportion of sporulation per unit of leaf area (Genet *et al.*, 1997).

DNA EXTRACTION

At the end of the leaf-disk assay, *P. viticola* lesions on untreated leaf-disks (control conditions) were frozen. After freeze-drying overnight, DNA was extracted from each "oil spot" according to the standard CTAB-phenol/chloroform method described by Zolan and Pukkila (1986) with a slight modification by Corio-Costet *et al.* (2011). After extraction, DNA was precipitated with isopropanol, the pellet was washed with 70% ethanol and resuspended in 50 µl of sterile water.

CYTOCHROME *B* CODING GENE SEQUENCE COMPARISON

Polymerase chain reaction (PCR) was performed in a 30 µl volume containing 1 µl of DNA diluted three-fold, 3 µl of 10x PCR buffer (Eurogentec), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each primer (COB F2:5'-gtggattcgccgttgataatc-3' & COB R2:5'-ccaaataatccagccgatac-3'), 0.25 U of Taq silverstar DNA (Eurogentec). The PCR program was a first denaturation step of 4 min at 96°C, followed by 35 cycles of 45 s at 96°C, 50 s at 50°C, 1 min at 72°C.
PCR products were sequenced by the Genewiz company. For comparison of the *cytb* gene between sensitive and resistant strains, the sequences were analysed using the Vector NTI software (version 10).

Field Trials

Three field trials were conducted in 2017 and 2018 in vineyard conditions to evaluate the practical consequences of the presence of resistant strains of *P. viticola*. All 3 trials were carried out in the Belchim facility in Fronton (Haute-Garonne department, South-West of France). In this vineyard, the presence of specific resistance to cyazofamid was previously confirmed in 2016 and 2017 by bioassays (included in the monitoring).
In all trials, the assessed parameters were pest severity (percentage area of the observed organ showing symptoms of disease) and incidence (percentage of organs showing symptoms), on leaves and bunches. In each case, 50 randomly chosen organs per elementary plot were assessed. The data was then tested statistically, excluding the untreated, using an analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparison test (5% alpha risk).

First trial (2017). This trial aimed at comparing the practical results of 3 different treatments as detailed in the table below.
The design was randomized complete blocks with 4 replicates and an elementary plot size of 10 plants. Six applications were then carried out from May 17th to July 13th (8 to 13 days interval), in conditions of high moisture (11 to 80 mm of rain between each application). The assessment was carried out on July 17th at BBCH 77-79.

Product	Active substance
Untreated	
Reference 1 (2 kg/ha)	zoxamid + mancozeb (123+1378 g/ha)
Mixture 1 (2.5 L/ha)	cyazofamid + folpet (100+1000 g/ha)
Experimental 1 (4 L/ha)	cyazofamid (100 g/ha)

Second trial (2018). This trial aimed at evaluating the efficacy of QiI and QioI in presence of strains resistant to both modes of action.
The design was randomized complete blocks with 3 replicates and an elementary plot size of 10 plants. Four applications were then carried out from May 17th to June 28th (14 days interval), in conditions of adequate moisture (31 mm of rain between aplications A and B, 77 between B and C, and 0.2 between C and D). The assement was carried out on July 10th at BBCH 75.

Product	Active substance
Untreated	
Reference 2 (2.5 kg/ha)	ametoctradin + metiram zinc (300+1100 g/ha)
Reference 3 (1.5 L/ha)	ametoctradin (300 g/ha)
IBE 3887 (4.5 L/ha)	cyazofamid + disodium phosphonate (112.5+1125 g/ha)
Experimental 1 (0.28 L/ha)	cyazofamid (112.5 g/ha)
Mixture 1 (2.5 L/ha)	cyazofamid g/ha + folpet (100+1000 g/ha)

Third trial (2018). This trial aimed at evaluating the practical efficacy of QiI and QioI in a spray program in presence of strains resistant to both modes of action.
The design was randomized complete blocks with 3 replicates and an elementary plot size of 10 plants. Three applications were then carried out, as described in the table above. The applications were carried out from May 17th to June 14th (14 days interval), in conditions of adequate moisture (31 mm of rain between aplications A and B, 77 between B and C). The assement was carried out on July 9th on bunches and July 10th on leaves at BBCH 75.

Application 1	Application 2	Application 3
Untreated		
Mixture 1 (2.5 L/ha): cyazofamid 100 g/ha + folpet 1000 g/ha	Reference 4 (3 kg/ha): fluopicolid 133.2 g/ha + fosetyl aluminium 2000 g/ha	IBE 3887 (4.5 L/ha): cyazofamid 112.5g/ha + disodium phosphonate 1125 g/ha
IBE 3887 (4.5 L/ha): cyazofamid 112.5g/ha + disodium phosphonate 1125 g/ha	Reference 4 (3 kg/ha): fluopicolid 133.2 g/ha + fosetyl aluminium 2000 g/ha	Mixture 1 (2.5 L/ha): cyazofamid 100 g/ha + folpet 1000 g/ha
IBE 3887 (4.5 L/ha): cyazofamid 112.5g/ha + disodium phosphonate 1125 g/ha	Reference 4 (3 kg/ha): fluopicolid 133.2 g/ha + fosetyl aluminium 2000 g/ha	Reference 2 (2.5 kg/ha): ametoctradin 300 g/ha + metiram zinc 1100 g/ha
Reference 2 (2.5 kg/ha): ametoctradin 300 g/ha + metiram zinc 1100 g/ha	Reference 4 (3 kg/ha): fluopicolid 133.2 g/ha + fosetyl aluminium 2000 g/ha	IBE 3887 (4.5 L/ha): cyazofamid 112.5g/ha + disodium phosphonate 1125 g/ha
Reference 5 (2.8 L/ha): copper (TBCS) 746.5 g/ha + zoxamide 112 g/ha	Reference 4 (3 kg/ha): fluopicolid 133.2 g/ha + fosetyl aluminium 2000 g/ha	Reference 6 (4 kg/ha): metiram zinc 1156 g/ha + fosetyl aluminium 1884 g/ha

RESULTS

MONITORING OF THE *P. VITICOLA* POPULATIONS' SENSITIVITY TO CYAZOFAMID

The monitoring allowed to detect some populations not perfectly controlled by cyazofamid even with SHAM (salicylhydroxamic acid) used as inhibitor of the AOX pathway. At this time, depending on their susceptibility to cyazofamid, *P. viticola* populations can be classified into 3 categories, either sensitive to this active substance (named S) or resistant by the activation of their AOX pathway (named R_{AOX}) or resistant, through a specific resistance putatively related to the target (named R_{cyazo}).

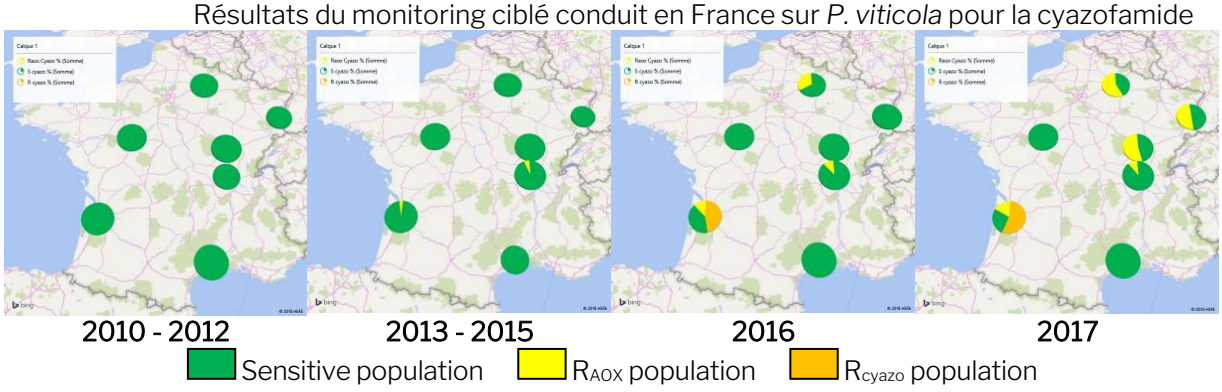
From 2010 to 2012: 159 samples were analysed and all were sensitive.

From 2013 to 2015: 197 samples were tested, and first occurrences of R_{AOX} strains were observed in 2 areas (South West and Beaujolais). In 2015, the first suspected case of specific resistance was observed.

In 2016: 106 samples were analysed and a specific shift of sensitivity (R_{cyazo}) was confirmed by bioassays in the Bordeaux/South-West area. At this time, further work was initiated to fully characterise the potentially resistant strains.

In 2017: 85 samples were analysed for their sensitivity to cyazofamid; presence of R_{AOX} strains was confirmed, especially in the East (Champagne, Burgundy, Alsace) and more marked presence of specific resistant strains in the South West of France (Bordeaux, Charentes, South-West) (Figure 1). Molecular analyses on R_{cyazo} populations provided the first evidence of the existence of a specific cyazofamid resistance with the detection of a mutation in the *cytb* gene in R_{cyazo} strains.

Figure 1 : *P. viticola* cyazofamid targeted monitoring results in French vineyard areas
Résultats du monitoring ciblé conduit en France sur *P. viticola* pour la cyazofamide



Additionally, using the same samples, bioassays were conducted with a QioI fungicide (ametoctradin) in 2017. Samples concerned by specific resistance (R_{ameto}) and R_{AOX} populations were found also for this mode of action (Figure 2).

Figure 2 : *P. viticola* cyazofamid/ametoctradin compared results in French vineyard areas
Résultats comparés du monitoring sur *P. viticola* pour la cyazofamide et l'amétoctradine

QiI QioI

Sensitive population R_{AOX} population Population with specific resistance (R_{cyazo}/R_{ameto})

IDENTIFICATION OF A MUTATION IN *CYTB* GENE ASSOCIATED TO THE RESISTANCE TO CYAZOFAMID IN THE LABORATORY

From populations characterized by contrasted sensitivity profiles to cyazofamid, S, R_{AOX} and R_{cyazo} single-sporangiophore *P. viticola* strains were isolated and subsequently tested again for their sensitivity to cyazofamid.

The results show that all sensitive strains tested are controlled at 1 mg/L of cyazofamid, R_{AOX} strains are still able to grow and sporulate in presence of cyazofamid but not when SHAM is added, which indicates that these strains are resistant to cyazofamid through the activation of their AOX pathway. Finally, all the R_{cyazo} strains tested are capable of developing in the presence of cyazofamid and SHAM. These latter results support the idea that R_{cyazo} strains are resistant to this active substance, caused by a mutation of the site targeted by cyazofamid in the mitochondria.

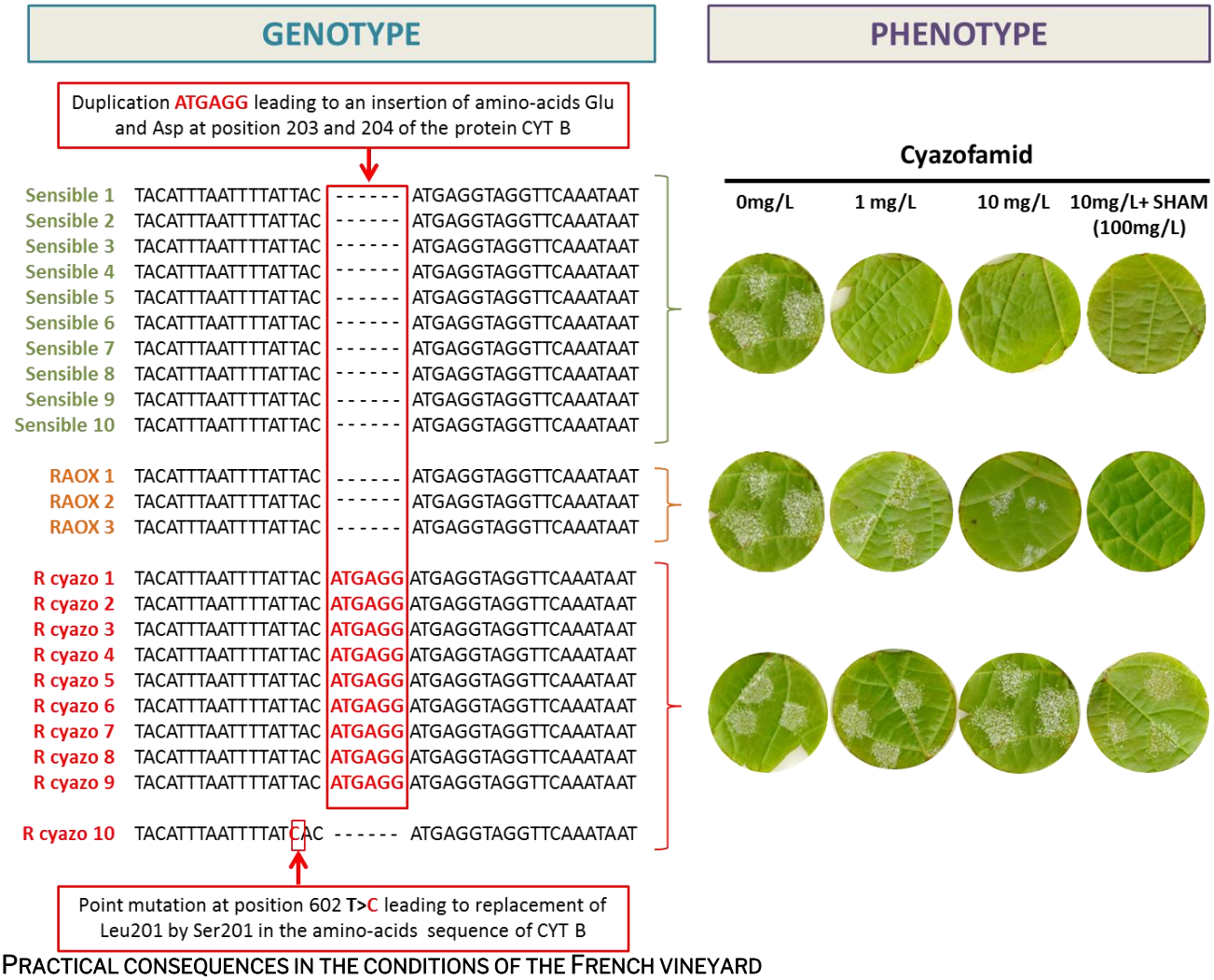
To confirm this hypothesis, total DNA was extracted from S, R_{AOX}, and R_{cyazo} strains in order to amplify by PCR the *cytb* gene sequence with specific primers.

The analysis of the *cytb* gene sequence revealed in R_{cyazo} strains a duplication of six base pairs at position 604 or 610, both leading to an insertion of 2 amino-acids (glutamic acid and aspartic acid) at the Qi site, behind the residue H202, identified as essential for the catalytic activity of the protein (Leroux *et al.*, 2015). Depending on if we consider the insertion in position 604 or 610 in the *cytb* gene sequence, this duplication can be named respectively H202-ED-E203, as illustrated in Figure 3, or E203-DE-V204 as already described in the national note.

On a side note, the R_{cyazo} phenotype was also associated to a point mutation on the *cytb* gene sequence at position 602 leading to the replacement of Leucine 201 by a Serine 201. This latter mutation was rare and detected only once.

The sequence of the *cytb* gene of the R_{AOX} strains is identical to that of the susceptible strains.

Figure 3: Comparison of the sequence of the gene coding for cytochrome *b* in sensitive, R_{AOX} and R_{cyazo} strains.
Comparaison de la séquence du gène codant le cytochrome *b* chez des souches sensibles, R_{AOX} et R_{cyazo}.

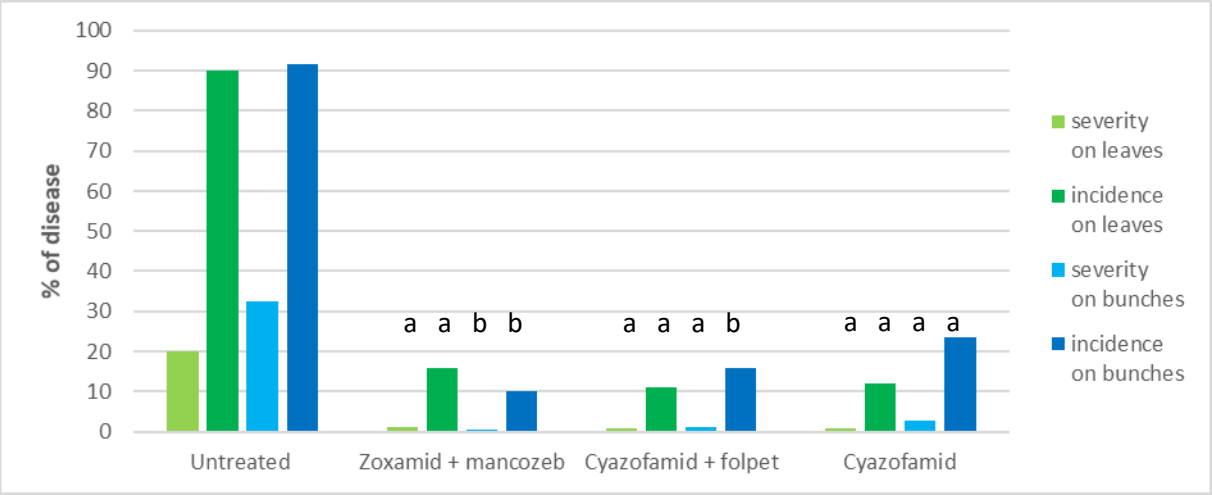


PRACTICAL CONSEQUENCES IN THE CONDITIONS OF THE FRENCH VINEYARD

After the first confirmed resistant strains detected in laboratory by using leaf-disk bioassays in 2016, it was decided to set up a field trial in 2017 in the presence of resistant strains, to assess the practical consequences of the cyazofamid resistant populations emergence.

When cyazofamid solo (100 g a.s./ha, equivalent to 4 L/ha of the commercial specialty IBE 3887) was compared to a standard reference and to a test mixture (cyazofamid+folpet, 100+1000 g a.s./ha), no significant difference was recorded on the leaves severity and leaves incidence (Figure 4). The cyazofamid solo formulation reached the same level of efficacy than the standard reference on leaves. On bunches, the control was significantly lower but remained high and satisfactory in these conditions of infection. It must be noted that this solo formulation (without disodium phosphonate) is not authorised for use on grapevine, and it is therefore not expected to behave as well as the established market references for this use. In this vineyard, the presence of resistant strains was confirmed even though it was not possible to quantify them within the population. This tends to suggest that the practical efficacy of cyazofamid (and thus the relevance of the formulated product in the control of downy mildew) was not affected under the conditions of this trial despite the presence of cyazofamid resistant populations.

Figure 4: Results obtained in the first field trial (2017)
Résultats obtenus en conditions pratiques (essai 1, 2017)



Further trials were carried out in 2018 for confirmation.
Cyazofamid solo was compared to mixtures with disodium phosphonate (IBE 3887) and with folpet (Figure 5). No significant difference was found, showing that cyazofamid solo was able to deliver a level control of the disease similar to other specialties. This was confirmed by the comparison with ametoctradin-based solutions: no sigificant difference appeared in this case either. All products failed to control successfully the incidence on bunches in the difficult conditions of this season, but the severity remained very low.
In the case of a spray program including only one or two applications of cyazofamid (Figure 6), the levels of efficacy obtained were even higher and no significant difference was detected between treatments.

Figure 5: Results obtained in the second field trial (2018)
Résultats obtenus en conditions pratiques (essai 2, 2018)

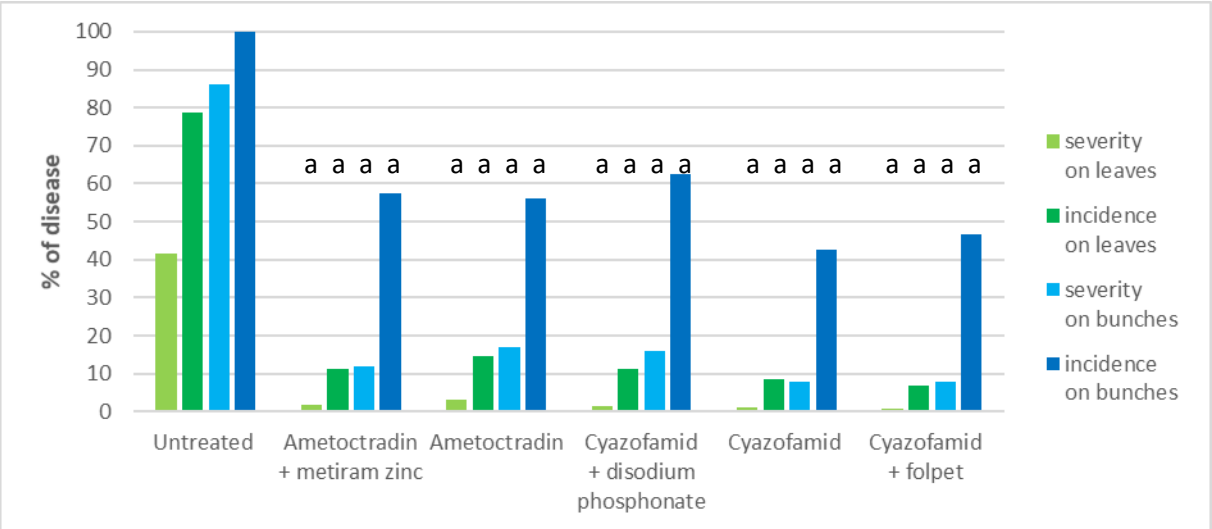
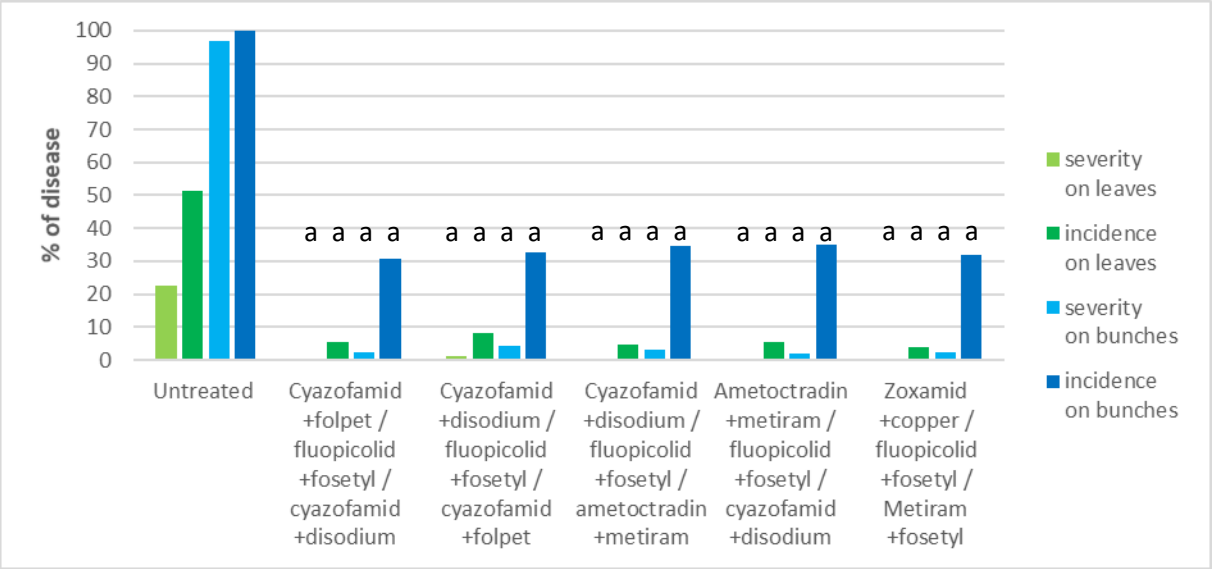


Figure 6: Results obtained in the third field trial (2018)
Résultats obtenus en conditions pratiques (essai 3, 2018)



DISCUSSION

EMERGENCE OF RESISTANT STRAINS

Clearly, some *P. viticola* strains have developed different adaptations in some regions of France: R_{AOX} is a non-specific adaptation allowing to activate an alternative repiratory pathway and leading to survival in the presence of various fungicides including QiI; and a specific resistance R_{cyazo} has been detected in France in 2016, mainly located in the Bordeaux/Charentes/South-West area.
It can be noted that our results show that QioI are concerned as well by the emergence of R_{AOX} and specific R_{ameto} populations. There is no evidence of positive cross-resistance between the two modes of action due to target site resistance, even if both compounds bind to the Qi site of the cytochrome *b*. Furthermore, the mechanism of R_{AOX} resistance remains poorly understood and there is no explanation for the fact that some samples were found to behave as R_{AOX} with one substance and sensitive with the other one.
Our results are not quantitative as the monitoring was performed in a targeted way (non-random). Therefore, the actual presence of resistant populations in the vineyard may not be as spread as our results might suggest. From 2018, the monitoring is now switched to random sampling, in order to be able to draw a more accurate picture of the situation.

IDENTIFICATION OF A MUTATION ASSOCIATED TO THE RESISTANCE

From resistant populations, single-sporangiophore strains have been isolated and the sequence of their cytochrome *b* gene has been analysed. Sequencing revealed that all tested strains able to grow and sporulate in presence of cyazofamid and SHAM display an insertion of six base pairs in *cytb* gene, leading to insertion of 2 amino-acids at the Qi site (E203-DE-V204). This mutation could potentially modify the structure of the cytochrome *b* at the Qi site and prevent the binding of cyazofamid. These results support the idea that specific resistance to cyazofamid is currently emerging.
Moreover, as expected, sequencing revealed that tested strains able to grow and sporulate in presence of cyazofamid but not when SHAM is added (R_{AOX}), display a *cytb* gene similar to sensitive strains.

PRACTICAL CONSEQUENCES OF THIS RESISTANCE

From the results obtained in three different field trials, we have determined that in the conditions of the South-West of France, and following good agricultural practices and the “Note Nationale” recommendation, the practical efficacy of cyazofamid (*i.e.* the performance of the commercial specialty IBE 3887) is expected to remain a very relevant tool of control of the grapevine downy mildew, even in presence of resistant strains in the vineyard.

CONCLUSION

Recognizing that the development of resistance in fungicides in general, and to cyazofamid in particular is a growing concern, an ambitious research program is being carried out by ISK and Belchim, in collaboration with Staphyt L&G.

After trials in 2017-2018, we decided for the future to continue to increase our level of knowledge and act in real condition on resistance management:

- Determine the fitness of the strains in winter conditions (survival rate);
- Evaluate the cross-resistance with other related fungicides, such as amisulbrom;
- Development of the PCR method: molecular tests to quickly detect R_{cyazo} isolates;
- Understanding the mechanism of resistance to cyazofamid can assist later the rapid screening of resistant populations using molecular tests (q-PCR) in order to counteract the emergence of fungicide resistance (2019-2020); using q-PCR method makes it possible to determine the frequency of resistant genotypes to cyazofamid and to further assess if there is any correlation with a potential lost of efficiency in the field.

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