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NEW FINDINGS ON THE AMETOCTRADIN MODE OF ACTION AND GRAPEVINE DOWNY MILDEW (*PLASMOPARA VITICOLA*) RESISTANCE TO COMPLEX III INHIBITORS

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ABSTRACT
Ametoctradin is the latest complex III inhibitor introduced in 2012 by BASF for the control of grapevine downy mildew. The binding site of ametoctradin has been described in 2016 (Fehr, *et al.* 2016) and has led to its classification as QoI-D or QoSI inhibitor. Recent studies have revealed that ametoctradin has a more complex mode of action than previously thought and can bind to both the Qo- and Qi-sites of the cytochrome b (Dreinert, *et al.*, 2018). These new findings will be put into perspective together with the latest results from the BASF resistance monitoring. The BASF view on the most appropriate resistance strategy for a sustainable management of the complex III inhibitors will be presented and discussed.

Keywords: Ametoctradin, Mode of action, Complex III inhibitors, Resistance management, Downy mildew.

RÉSUMÉ
NOUVELLES CONNAISSANCES SUR LE MODE D'ACTION DE L'AMÉTOCTRADINE ET LA RÉSISTANCE DU MILDIOU DE LA VIGNE AUX INHIBITEURS DU COMPLEXE III
L'amétoctradine, introduit en 2012 par BASF, est le plus récent inhibiteur du complexe III pour le contrôle du mildiou de la vigne. Le site de fixation de l'amétoctradine a été décrit en 2016 (Fehr, *et al.* 2016) ce qui lui a valu une classification dans le groupe des inhibiteurs QoI-D ou QoSI. Cependant, de récentes études ont démontré que l'amétoctradine a un mode d'action bien plus complexe qu'annoncé précédemment et peut se lier aux deux sites Qo et Qi du cytochrome b (Dreinert, *et al.*, 2018). Les connaissances novatrices sur ce mode d'action seront présentées et mises en parallèle avec les derniers résultats du suivi des résistances aux inhibiteurs du complexe III réalisé par BASF. Les préconisations de BASF concernant les stratégies les plus appropriées pour une gestion durable des inhibiteurs du complexe III seront également présentées et discutées.

Mots-clés : Amétoctradine, Mode d'action, Inhibiteurs du complexe III, Gestion de la résistance, Mildiou de la vigne.

INTRODUCTION

Inhibitors of the mitochondrial complex III are well known active ingredients used in agriculture and in medicine for the control of different diseases (*e.g.* atovaquone for *Plasmodium* or pyraclostrobin for Oomycetes). At the molecular level, they can bind to different positions within complex III: the Qo-site, the Qi-site or to both sites like the naturally occurring coenzyme ubiquinol. Fungicides acting as complex III inhibitors, such as ametoctradin, are very effective tools to control *Plasmopara viticola*, the causal agent of grape downy mildew (Table 1).

Table I: Summary of complex III inhibitors used in agriculture to control *Plasmopara viticola*
Liste des fongicides inhibiteurs du complexe III utilisés pour lutter contre *Plasmopara viticola*

FRAC Group	Mode of action	Chemical family	Active ingredient
C3	QoI	Methoxy-acrylates	Azoxystrobin
		Methoxy-carbamates	Pyraclostrobin
		Oxazolidinediones	Famoxadone
		Imidazolinones	Fenamidone
C4	QiI	Sulfamoyl-triazoles	Amisulbrom
		Cyano-imidazole	Cyazofamid
C8	QoSI	Triazolo-pyrimidylamines	Ametoctradin

Ametoctradin belongs to the triazolo-pyrimidylamine chemical family. It is known to bind at the Qo site stigmatellin binding subsite of the complex III in the respiratory chain (Fehr, *et al*., 2016). The FRAC group (Fungicide Resistance Action Committee) classified this active ingredient (a.i.) in the QoSI group (Quinone-outside-Stigmatellin-like-Inhibitor). Ametoctradin inhibits several essential biological processes of Oomycetes such as zoospore formation, release or germination.

P. viticola is classified by FRAC as a plant pathogen that has a high risk to develop fungicide resistance (Brent, 1998). All active ingredients targeting the complex III are single-site inhibitors, for which single mutations can cause specific resistance and consequently a loss of activity. Besides the classical risk of selection of target-site resistance, another adaptive mechanism that affects equally all complex III inhibitors is the overexpression of the alternative oxidase (AOX) enzyme, involved in a alternative respiration pathway. Several resistance mechanisms to complex III inhibitors were already identified in *P. viticola* and published in the "Note Technique Commune" (Note commune, 2018) (Table II).

Table II: Known *P. viticola* resistance mechanisms affecting complex III inhibitors
Mécanismes de résistance de *P. viticola* aux fongicides inhibiteurs du complexe III

	QoI	QiI	Ametoctradin
Specific resistance	G143A F129L	L201, Insertion 1 (E203-DE-V204), Insertion 2 (BASF finding, not published)	S34L
Unspecific resistance	AOX overexpression		

Knowledge on the specific resistance to Qo-site inhibitors is well established but the knowledge on the target-site resistance mechanisms affecting QiIs and ametoctradin is more recent (Dreinert, *et al*., 2018).

After the discovery of the binding mode of ametoctradin (QoSI) by BASF in 2016 (Fehr, *et al*., 2016), some observations in the laboratory and the detection of the first ametoctradin-resistant isolates led BASF researchers to continue the investigation work on the complex ametoctradin mode of action.

In this context, our study aimed to:

i) Deepen our understanding of ametoctradin binding to the complex III
ii) Understand the cross-resistance relationships existing among complex III inhibitors
iii) Monitor the evolution of *P. viticola* sensitivity towards complex III inhibitors, in order to provide to vine growers the most appropriate anti-resistance management recommendations.

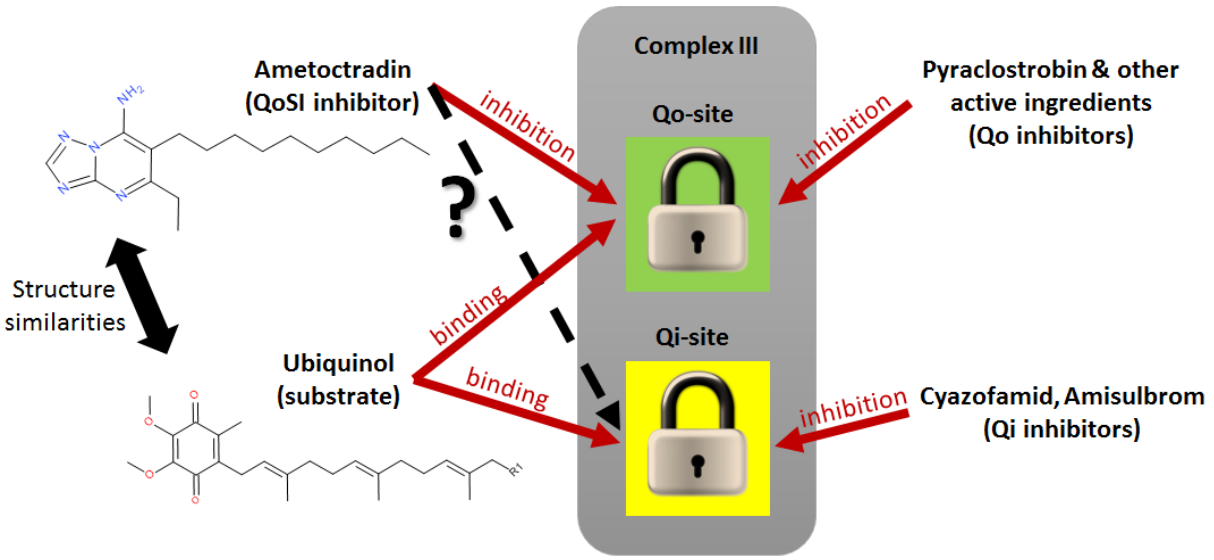
NEW FINDINGS ON THE AMETOCTRADIN MODE OF ACTION

MATERIAL AND METHODS

Similarities were observed between the 3D structure of ametoctradin and the substrate ubiquinol, that is able to bind to both the Qo- and Qi-site within complex III. Laboratory experiments were set up to determine whether ametoctradin can also bind to both sites of cytochrome b, as modeling studies suggested an ubiquinol-like binding mode for ametoctradin (Figure 1).

Figure 1: Binding mode of ametoctradin and other complex III inhibitors
Mode de fixation de l'amétoctradine et des autres inhibiteurs du complexe III

Spectrophotometric and molecular binding studies were conducted on *Pythium* (oomycete) and

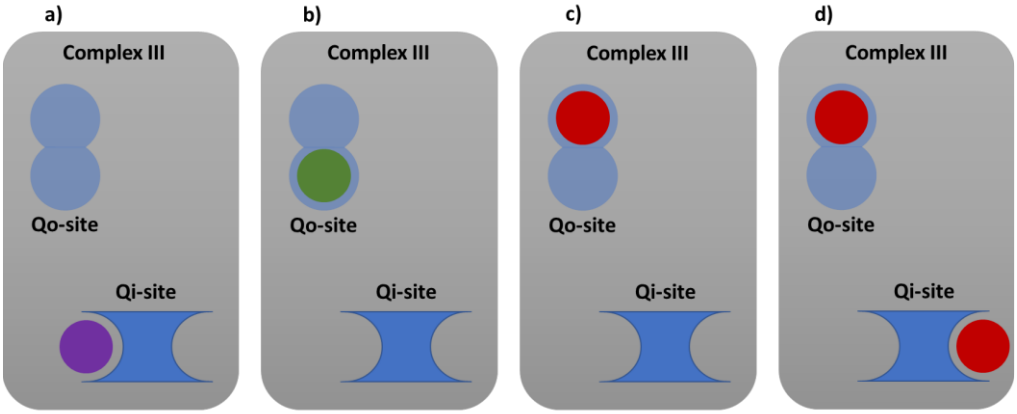


Saccharomyces cerevisiae (yeast) to investigate how the complex III of each species acts when exposed to the inhibitors ametoctradin (QoSI), cyazofamid (QiI) and amisulbrom (QiI) (Dreinert, *et al.*, 2018).

RESULTS & DISCUSSION

The results of the molecular assays revealed that ametoctradin, in addition to binding to the Qo-site, can also bind to the Qi-site of the cytochrome b in the respiratory chain. The study also demonstrates that ametoctradin binds to the Qi-site in a different way compared to cyazofamid and amisulbrom, as shown on Figure 2 (Dreinert, *et al.*, 2018).

Figure 2: Model illustrating the binding modes of different complex III inhibitors
Modélisation des modes de fixation de divers inhibiteurs du complexe III



Qi-Inhibitors (QiI) Qo-Inhibitors (QoI) QoS-Inhibitors (QoSI) Ametoctradin

Figure 2a represents the binding mode of Qi inhibitors, such as antimycin A, amisulbrom and cyazofamid, binding to the Qi-site of the complex III. Figure 2b shows how Qo-inhibitors, such as strobilurins, myxothiazol, fenamidone and famoxadone, bind to the proximal domain of Qo-site. Figure 2c shows the binding mode of QoS-class inhibitors (UHDBT, stigmatellin) on the distal domain of the Qo-site. Finally, Figure 2d gives an insight into the binding mode of ametoctradin to both the Qo- and Qi-sites of the complex III.

Spectrophotometric studies conducted by BASF (Dreinert, *et al.*, 2018) have also demonstrated that the binding mode of ametoctradin is different from the one of classic Qi inhibitors like cyazofamid and amisulbrom as shown on Figure 2.

RESISTANCE OF *PLASMOPARA VITICOLA* TO COMPLEX III INHIBITORS

FOREWORD

P. viticola evolved both specific and non-specific resistance to complex III inhibitors. Thus, the issues of both the potential cross-resistance patterns among the different a.i. and the evolutionary dynamics of *P. viticola* populations are raised.

CROSS-RESISTANCE RELATIONSHIP BETWEEN AMETOCTRADIN & CYAZOFAMID

Material & methods

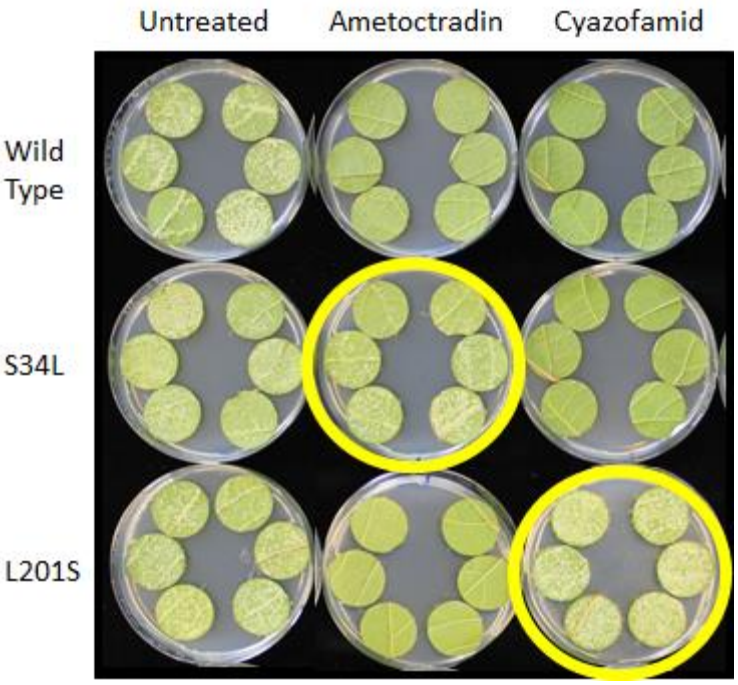
A leaf disc assay was conducted in Petri dishes. Two mutant genotypes of *P. viticola* were assessed, carrying either the substitution S34L (affecting ametoctradin) or L201S (affecting cyazofamid) in cytochrome b. Leaf discs were treated with ametoctradin (10 ppm, discriminatory dose) or cyazofamid (10 ppm, discriminatory dose), then inoculated with the mutant genotypes and finally incubated to assess the inhibition of sporulation. The wild type genotype was used as the sensitive reference to assess the potential cross-resistance pattern among ametoctradin and cyazofamid (QiI) due to the two point mutations. The pathogen sporulation was visually scored by 0 (inhibition of the sporulation) or 1 (sporulation occurred).

Simultaneously, a zoospore release test was carried out with ametoctradin (10 ppm) and cyazofamid (10 ppm) on five *P. viticola* strains including two wild type strains and three resistant strains (S34L-, L201S- and AOX-based resistance). The percentage of efficacy of these two a.i. was calculated based on the inhibition of zoospore release (empty sporangia = zoospores were released) at the a.i. concentration of 10 ppm.

Results & discussion

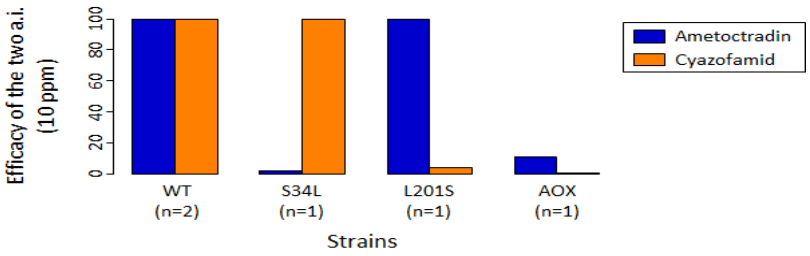
Regarding the leaf disc test, sporulation was observed in all untreated controls for each isolate (untreated, S34L and L201S) as expected. Sporulation was not inhibited by ametoctradin or by cyazofamid at 10 ppm in the isolates carrying the mutation S34L or L201S, respectively (Figure 3).

Figure 3: Leaf disc test to assess the cross-resistance pattern among ametoctradin and cyazofamid Test des disques foliaires pour analyser le pattern de résistance croisée entre l'amétoctradine et la cyazofamide



Regarding the zoospore release test, both ametoctradin and cyazofamid were able to inhibit the sporulation for the two wild type strains, as expected (Figure 4).

Figure 4: Efficacy of ametoctradin and cyazofamid on sensitive (WT) and resistant *P. viticola* strains in zoospore release tests
Efficacité de l'amétoctradine et de la cyazofamide sur des souches sensibles (WT) et résistantes de *P. viticola* pour les tests de sporulation



The presence of the S34L substitution affected the efficacy of ametoctradin only. The L201S substitution affected the efficacy of cyazofamid only. None of these substitutions determined positive cross-resistance between ametoctradin and cyazofamid. Only the populations overexpressing the AOX were not affected by both activeingredients.

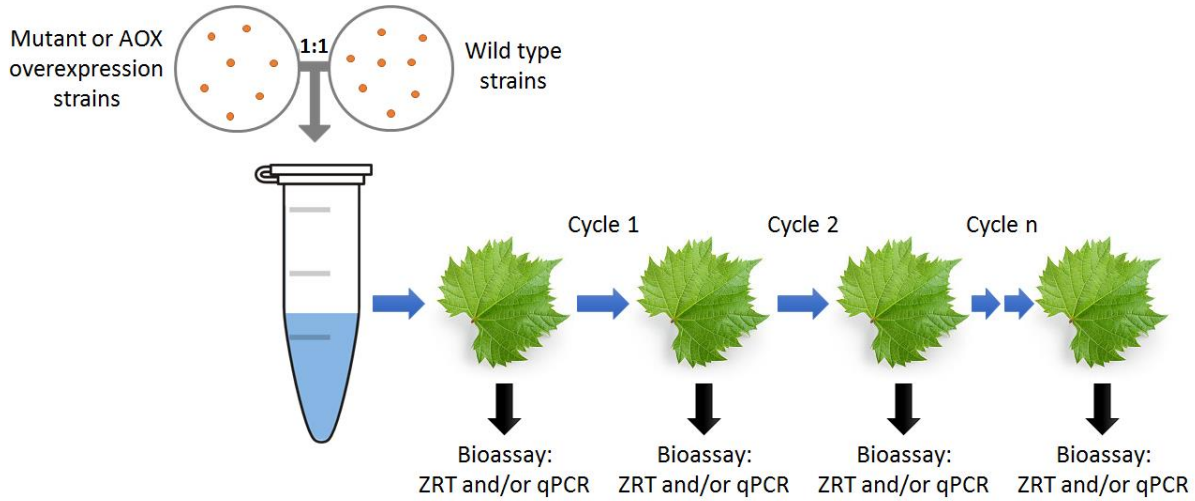
FITNESS OF *PLASMOPARA VITICOLA* RESISTANT ISOLATES

Material & methods

The fitness of sensitive (wild type) strains and S34L or AOX strains was tested by a competitive co-cultivation of these isolates on detached leaves in the greenhouse without selection pressure (untreated leaves) as shown on Figure 5. Wild type isolates were either mixed with S34L strains or AOX strains in a 1:1 ratio. The mixture was inoculated on the leaves and the sporulation occurred 1

week after. Sporangia were then harvested after each cycle and the frequency of sporangia with S34L or AOX overexpression were determined in a zoospore release test (ZRT on Figure 5) and/or a qPCR assay (molecular tool for the quantification of the S34L mutation only). A total of 8 cycles was carried out.

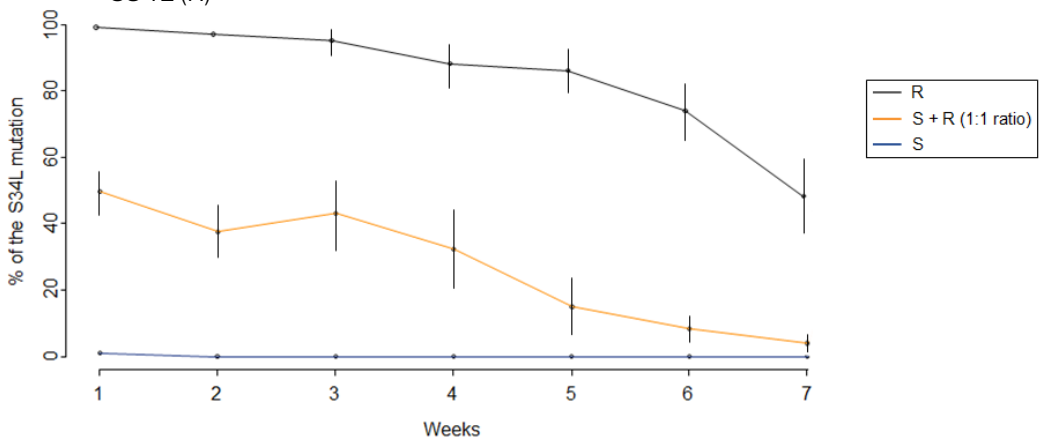
Figure 5: Methods used to assess the fitness of wild type strains *versus* mutant or AOX strains of *P. viticola*
Méthodes utilisées pour étudier la fitness de souches de *P. viticola* sensibles *versus* souches mutantes ou surexprimant l'AOX



Results & discussion

The results of the competition tests performed in the laboratory with the strains carrying the S34L substitution suggested a reduced fitness for the resistant strain (S+R on Figure 6) compared to the wild-type sensitive strains. The competitiveness of this type of strain seems low after seven cultivation cycles. A slower development of these strains is therefore expected.

Figure 6: Fitness study of one sensitive strain(S) and one strain carrying the S34L mutation (R)
Etude de la fitness d'une souche sensible (S) et d'une souche portant la mutation S34L (R)



Regarding the AOX-related competition study, no change was observed in the two control experiments including strains with or without AOX overexpression (Fehr, et al., 2016). However, the percentage of AOX-overexpressing strains was drastically reduced after 8 cycles in presence of sensitive strains without any fungicide application (Fehr, et al., 2016). The results suggested a reduced competitiveness of AOX-overexpressing strains (Fehr, et al., 2016). Indeed, the alternative respiration is a highly energy-consuming mechanismsensitivity of *Plasmopara viticola* populations to complex III inhibitors in France

Material & methods

DNA was extracted from 102 *P. viticola* populations sampled in 2017 by an independent contractor in 9 French grape growing regions (Table III). A molecular tool (Quantitative PCR – qPCR) was used to quantify G143A-, S34L-, Ins-1- (E203-DE-V204), Ins-2- and L201S-mutant alleles conferring resistance to Complex III inhibitors (Table 2). AOX-based resistance was quantified using SHAM (100 mg/L) to decipher whether the strains overexpressed the AOX enzyme.

Table III Number of *P. viticola* populations sampled in each French vineyard
Nombre de populations de *P. viticola* échantillonnées dans chaque vignoble en France

Regions	Armagnac	Bordeaux	Burgundy	Champagne	Cognac	Languedoc	Rhône Valley	Savoie	Loire Valley
Nb of *P. viticola* populations sampled	16	9	12	14	12	9	13	3	14

Results & discussion

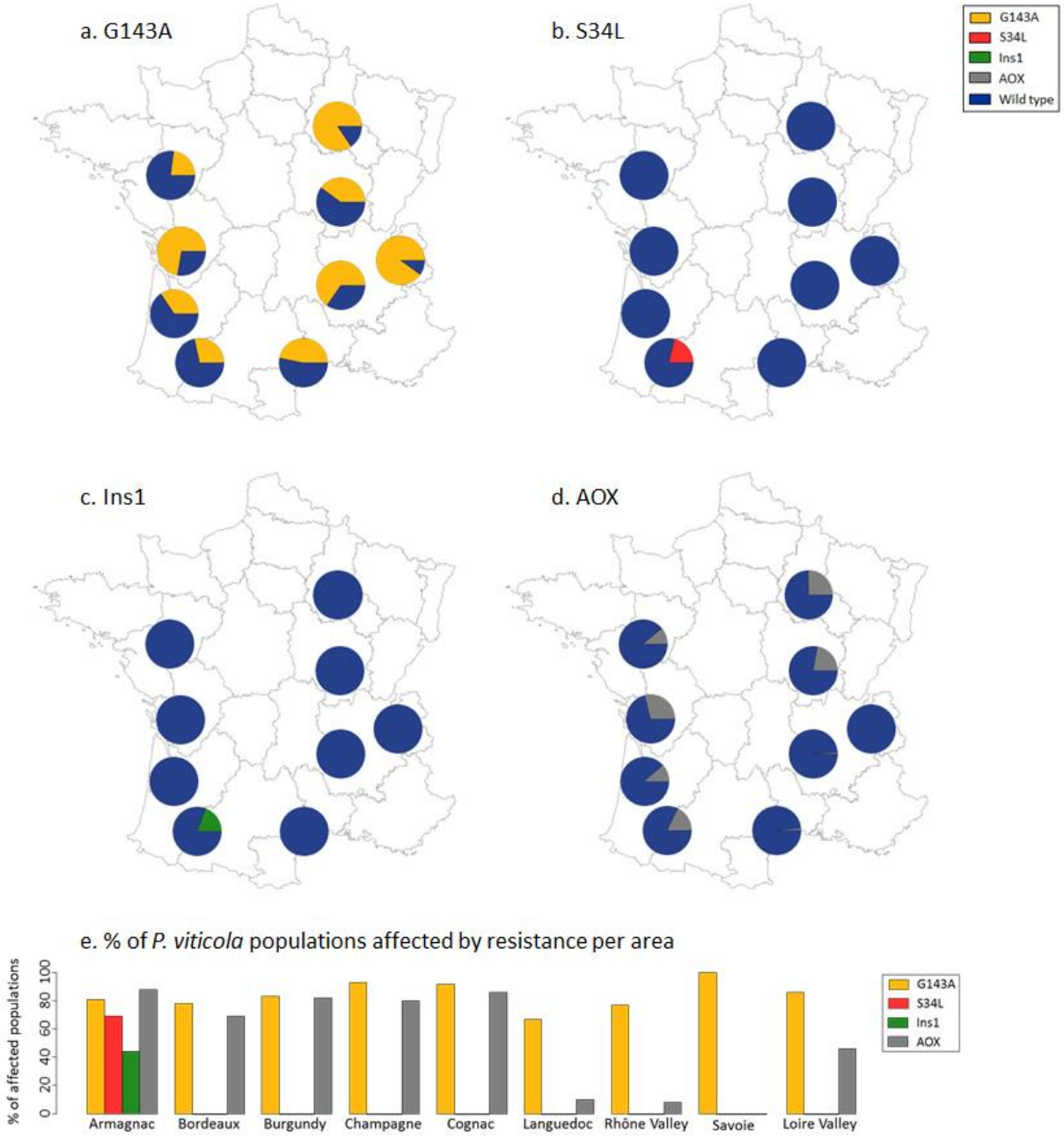
Armagnac was the only vineyard in which the substitutions G143A, S34L,Ins1 and the AOX overexpression were identified (Figure 8). The Ins2 and L201S substitution, previously detected in another European country (BASF data, not published) were not found in the 102 tested populations. In Armagnac, *P. viticola* populations could be more genetically diverse and their evolutionary dynamics may explain our observations.

The substitution G143A, conferring resistance to Qo inhibitors, was found in all sampled regions (Figure 8a). The frequency of G143A ranged from 28% (Armagnac) to 90% (Savoie) and this change was detected in more than 60% of the tested populations per area (Figure 8e). This high level of resistance is not so surprising as Qo inhibitors have been commonly used for more than 20 years to control grape downy mildew and resistance cases were reported since the early 2000s (Corio-Costet, *et al*., 2009).

Regarding the substitutions S34L (conferring specific resistance to ametoctradin) and Ins1 (conferring specific resistance to Qi-inhibitors), their frequencies reached 21% and 19% in Armagnac, respectively (Figure 8b-c). In addition, insertion-based mutations have no impact on ametoctradin activity (BASF data, not published). In 2017, specific resistance to ametoctradin was localized at a low level in Armagnac only.

Unspecific resistance due to the overexpression of AOX was observed in all sampled regions, except in Savoie (Figure 8d), at variable frequencies ranging from 1% (Rhône Valley, one population affected) to 29% (Cognac, 12 populations affected). This non-target-site mechanism can affect all complex III inhibitors (Note commune, 2015).

Figure 8: Specific and unspecific resistance of *P. viticola* populations to Complex III inhibitors and frequencies of populations affected for each vineyard (BASF monitoring – 2017)
Résistances spécifique et non spécifique des populations de *P. viticola* aux inhibiteurs du complexe III et fréquences des populations touchées pour chaque vignoble (BASF monitoring – 2017)



EVOLUTION OF BOTH SPECIFIC AND NON SPECIFIC RESISTANCE OVER TIME

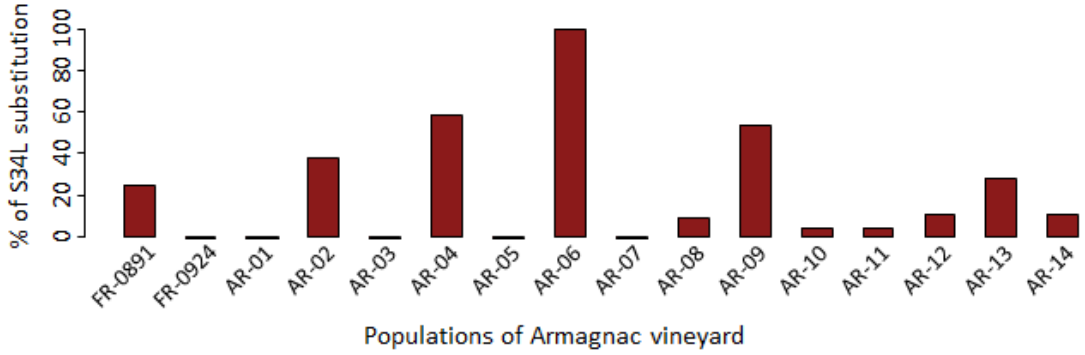
Material & methods

A resistance monitoring was set up by BASF to study the evolution of specific and unspecific resistance of *P. viticola* populations to complex III inhibitors (QoI, QiI, QoSI and AOX) in French vineyards. Herein, we focused on the evolution of S34L- and AOX-based resistance. The presence of S34L mutant alleles was quantified using qPCR and the overexpression of AOX was estimated using SHAM (100 mg/L).

Results & discussion

The S34L substitution was only identified in the Armagnac region (Figure 8b). The frequencies of *P. viticola* populations affected by S34L-based resistance increased over time in this region, ranging from 10% in 2015 to 50% in 2017 (data not shown). Nevertheless, since the first case reported in 2015, the frequency of S34L-mutant alleles is moderate within the sampled populations: 80% of *P. viticola* populations in Armagnac showed frequencies of S34L mutant alleles under 40% in 2017 (Figure 9).

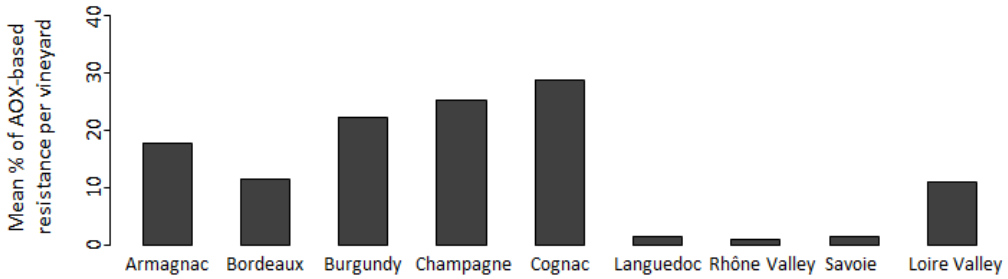
Figure 9: Frequency of the S34L substitution affecting cytochrome b in *P. viticola* populations in Armagnac (2017)
Fréquence de la substitution S34L affectant le cytochrome b dans les populations de *P. viticola* en Armagnac (2017)



Our results could be explained by both the fitness penalty of this specific resistance and the appropriate resistance management conducted by grape growers, following BASF's recommendations and those specified in the Note Commune (Note commune, 2017).

From 2011 to 2015, frequencies of the AOX-based resistance were very low, ranging from 0% to 7% in all vineyards (data not shown). Since 2015, despite the increase of populations affected by this resistance mechanism (especially in Armagnac), the level of resistance within populations stayed moderate (Figure 10).

Figure 10: Mean frequency of AOX-based resistance per vineyard (2017)
Fréquence moyenne de la résistance non spécifique dûe à la surexpression de l'AOX par vignoble (2017)



As specified previously, this unspecific resistance mechanism may affect the competitiveness of AOX-strains in absence of fungicides treatments selecting for resistance. This monitoring should still be carried out within the next years to deepen our understanding of the factors contributing to such variability of AOX-based resistance frequencies.

CONCLUSION AND PRACTICAL RECOMMENDATIONS FOR THE GROWERS

Ametoctradin is a double-site inhibitor which binds on both the Qo- and Qi-site of the complex III. Ametoctradin binds to the Qi-site in a different way compared to other Qi inhibitors such as cyazofamid, which explains why no cross-resistance was observed between these two active ingredients. Fitness studies on S34L and AOX strains suggested that the evolution of resistance could be slow in the absence of selection pressure and resistance management in the field can contribute even more to slow down the development of resistance to complex III inhibitors. The specific characteristics of ametoctradin should be taken into account to provide appropriate anti-resistance management recommendations to grape growers.

BASF recommendations in France are based on key elements for the control of *P. viticola*:

1. General use of ametoctradin:
    a. Maximum two applications per year
    b. Preventive use of all ametoctradin containing products (*e.g.* ENERVIN, RESPLEND)
    c. Association with multisites or other a.i. efficient against *P. viticola*
    d. Alternation of modes of action
2. Because of non-specific resistance (AOX), we recommend:
    a. A maximum of two applications of QoSI and/or QiI containing products in alternation with other modes of action

BASF recommendations, associated to the Note Commune recommendations, help to provide a sustainable spray program to grape growers.

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