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Control strategies against grey mould (*Botrytis cinerea* Pers.: Fr) and corresponding fungicide residues in grapes and wines

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This study examines the most effective anti-*Botrytis* strategies leading to possible lower pesticides residues in wine. To provide wine growers with a number of high-quality solutions for protection against *Botrytis* for their vineyards while minimizing pesticide residues in the final product, various treatment approaches were tested. A total of 10 strategies with different specific fungicide treatments for controlling *Botrytis cinerea* were applied to grapes at different growing stages: flowering, bunch closure and colour change. The type of vine chosen was Gamay, as it is very sensitive to *Botrytis cinerea*. In each experimental plot, disease incidence and severity were assessed at harvest. In addition, pesticide residue analysis was carried out on grapes, musts and wines to monitor residue levels in each treatment and to follow changes at each stage of the wine-making process. A correlation was established between the efficiency of anti-*Botrytis* fungicide treatment and pesticide residues in wine. Several strategies using various fungicides showed good results in terms of treatment efficiency while minimizing pesticide residues in wine, thus providing interesting alternatives to limit the development of fungal resistance.

Keywords: pesticides; fungicide residues; grapevine; wine; *Botrytis cinerea*; grey mould

Introduction

Insecticides and fungicides are used to control pests and diseases in grapevines (fungi or insects). In viniculture, the most common pesticides are fungicides, used to control, for example, downy mildew, powdery mildew and grey mould caused, respectively, by *Plasmopara viticola*, *Erysiphe necator* and *Botrytis cinerea* fungi. *Botrytis cinerea* is a fungal pathogen occurring particularly frequently in Europe and especially in countries with cold wet climates, such as Switzerland or central and eastern France. *Botrytis* control is a major challenge, as grey mould can very rapidly destroy grape bunches and lead to subsequent defects in the wine. In addition, it is necessary to regularly alternate treatments, either with different mechanisms of action and/or different appropriate pesticide mixtures, to maintain efficiency and avoid fungal resistance. Thus, at harvest, it is possible to find various pesticide residues in grapes. Some of these residues pass through the technological process of wine production and are still present in the final product (Edder and Ortelli 2005). Several studies on the fate of pesticide residues from grapes to wine have been published (Cabras and Angioni 2000; Cabras et al. 2001). These studies have shown that some substances, such as penconazole, fluazinam, kresoxim-methyl and organophosphorous insecticides, disappear quickly from the grapes after treatment, whereas a number of pesticides have longer degradation times ($t_{1/2}$ 10–30 days, 57 days for pyrimethanil) and are still present at harvest. Pesticide behaviour during the wine-making process has also shown that, after fermentation with or without skins, pesticide residue levels in wine usually decrease compared to those on grapes or in must. However, some pesticides, such as azoxystrobin or pyrimethanil, have no preferential partition between the liquid (must) and the solid phases (lees and cake), and the concentrations measured in wines were similar to those on grapes. Among clarifying substances (bentonite, charcoal, gelatine, polyvinylpolypyrrolidone, potassium caseinate and colloidal silicon dioxide), only charcoal guaranteed total removal of most pesticide residues (Cabras and Angioni 2000; Cabras et al. 2001). Therefore, if grapes contain high levels of some types of pesticides, these can survive to the wine-making process and contaminate the final product.

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Human health and protection from exposure to pesticide residues in foodstuffs is a major objective. Thus, to ensure food safety for consumers, legislation, such as the EU directives (76/895/EEC, 86/362/EEC, 86/363/EEC and 90/642/EEC), or Swiss regulations (OSEC 1995) and Codex Alimentarius of FAO/WHO have established maximum residue limits (MRLs) for pesticides in foodstuffs and in wine.

In a survey of pesticide residues in wine (Edder and Ortelli 2005), approximately 250 wines of various origins were analysed by a LC–MS/MS method, using a simplified extraction procedure to complement traditional GC pesticides determination, to provide effective monitoring and control. The results showed that, although almost all wines were compliant with Swiss legislation, which is similar to the EU Directives, pesticide residues were nevertheless found in almost all 250 samples. Carbendazim, fenhexamid, azoxystrobin, cyprodinil, pyrimethanil, tebuconazol, dimethomorph, myclobutanil, thiophanate-methyl, carbaryl, iprovalicarb and fludioxonil were the substances most commonly found and were present in 3–69% of the samples. However, residue levels were between 10 and 50% of the MRLs (Edder and Ortelli 2005). In modern grape growing, many different treatments are used and, consequently, many active substances are found in wine; up to 10 different active compounds have been detect in wines.

In grapevines, the most commonly used compounds are fungicides to control downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*) and grey mould (*Botrytis cinerea*). Worldwide, *B. cinerea* cause one of the most serious fungal diseases on plants, including bunch rot of grapes. The control of *Botrytis* is a major challenge, as it can very rapidly destroy grape bunches, resulting in high economic losses and reduced wine quality. Control strategies have to consider the risk of resistance development in the pathogen. To prevent adaptation of the fungus to the active ingredients, only one application per year with products from the same chemical family are permitted in Switzerland. The first treatment can be applied at the end of bloom. For penetration of the active substances in the rachis, the most important time is before bunch closure. A third time is when berries change colour, at the so-called véraison, corresponding to mid-August in the North hemisphere. For best possible control of the disease, grape growers spray the specific products only in the bunch area and predominantly at véraison. Viticulturists regularly request authorization to spray at a later date, in accordance with a delay in harvest, as with other crops, etc. These practices could have a negative impact on the residues of active ingredients in the grape and wine, and their efficiency is not proven. Therefore, a detailed study of various effective anti-*Botrytis* strategies, leading to the lowest residues in the final product while maintaining effectiveness in control of grey mould, is important.

The objective was to test different treatment approaches allowing winemakers to obtain a quality product, without incurring significant economic losses due to grey mould, and to minimize pesticide residues for the consumer. During the study, several fungicides were used at three developmental stages of grape: flowering, bunch closure and at color change or ''véraison''. Culture, harvest and wine-making took place at Agroscope Changins-Wädenswil; residue analyses were performed in the laboratory of the Food Control Authority of Geneva.

Experimental

Anti-Botrytis *treatments*

The experiments took place at Changins (Nyon, Switzerland), where specific fungicides were applied to control *Botrytis cinerea* on Gamay grapes (clone 5–44 grafted on rootstock 3309; row distance: 1.15 m, distance between blocks: 2 m) at flowering (A, 22 June), at bunch closure (B, 11 July) and at colour change or ''véraison'' (C, 15 August), as presented in Table 1. The experimental design corresponded to a practical approach with unit blocks of 70 m^2 on two vine rows, sprayed on both side of the leaf canopy with an axial fan-sprayer (Fischer), mounted on a small Caterpillar tractor. Calibration of the sprayer was performed according to the Caliset method (Raisigl and Felber 1991; Viret 1999). The control of downy and powdery mildew was standard practice, with the fungicides having no side-effect on *Botrytis*. Management of the vines was in accordance with Swiss integrated production (www.vitiswiss.ch), with regulation of crop amount to 1 kg m^{-2} by green vintage. In each experimental plot, disease incidence (average percentage number of infected bunches) and severity (average estimated diseased bunch surface in %) were assessed at harvest on four replicates of 50 randomly selected bunches, according to the following scale: 0, $1/10 \leq 0.1$, $1/4 \leq 0.25$, $1/2 \leq 0.5$, $3/4 \leq 0.75$, of diseased bunch area (Müller and Schwinn 1992). As a comparison, the untreated, resistant cultivar Gamaret was assessed for grey mould in the same plot. Mean values with standard deviation were calculated for disease incidence and severity. At harvest, 1.5 kg of grape berries per plot was collected randomly for analyses of residues.

Must and wine makings

For each plot, all grapes were harvested separately and processed in a standard way to make red wine. Bunches were crushed, the rachis separated from the pulp and juice, and inoculated with standard yeasts for alcoholic fermentation during maceration.

Table 1. Specific fungicide treatments applied at different time to control grey mould (*Botrytis cinerea*).

Assay number	(A) Flowering [22 June 2006]	(B) Bunch closure [11 July 2006]	(C) Colour change or véraison [15 August 2006]
			
1	Folpet (2 kg ha^{-1})	Pyrimethanil (2.4 kg ha^{-1})	Boscalid (1.2 kg ha^{-1})
2	Folpet (2 kg ha^{-1})	Mepanipyrim (1.2 kg ha^{-1})	Trifloxystrobin (0.3 kg ha^{-1})/ dichlofluanid (2.4 kg ha^{-1})
3	Folpet (2 kg ha^{-1})	–	Cyprodinil/fludioxonil (1.2 kg ha^{-1})
4	Folpet (2 kg ha^{-1})	Trifloxystrobin (0.3 kg ha^{-1})/ dichlofluanid (2.4 kg ha^{-1})	–
5	Folpet (2 kg ha^{-1})	Fenhexamide (1.5 kg ha^{-1})	Cyprodinil/fludioxonil (1.2 kg ha^{-1})
6	Folpet (2 kg ha^{-1})	Cyprodinil/fludioxonil (1.2 kg ha^{-1})	–
7	Folpet (2 kg ha^{-1})	Fenhexamide (1.5 kg ha^{-1})	Trifloxystrobin (0.3 kg ha^{-1})/ dichlofluanid (2.4 kg ha^{-1})
8	Folpet (2 kg ha^{-1})	Cyprodinil/fludioxonil (1.2 kg ha^{-1})	Fenhexamide (1.5 kg ha^{-1})
9	Fenhexamide (1.5 kg ha^{-1}) Folpet (2 kg ha^{-1})	Cyprodinil/fludioxonil (1.2 kg ha^{-1})	–
10	Folpet (2 kg ha^{-1})	Cyprodinil/fludioxonil (1.2 kg ha^{-1})	Trifloxystrobin (0.3 kg ha^{-1})/ dichlofluanid (2.4 kg ha^{-1})

After 10 days, the juice was separated from the marc and pressed with a pneumatic press. The juice was further processed for malo-lactic fermentation by inoculating the young wine with *Leuconostoc oenos* bacteria, filtered and stabilised with sulphites. Samples of 250 ml per treatment for residue analyses were taken after crushing the berries for the must, after alcoholic fermentation for the young wine and before bottling the wine as final product.

Analytical procedure

To screen a large number of pesticides, two different multi-residues methods were used.

GC procedure for non-polar pesticides (unpublished)

A total of 99 non-polar pesticides, such as organochlorine and organophosphorous fungicides and insecticides, were extracted from samples with 100 ml acetonitrile and shaken mechanically for 30 min. After addition of 300 ml water and 10 ml of a saturated NaCl solution, pesticides were extracted by liquid–liquid extraction with 50 ml hexane. The organic phase was collected and evaporated to dryness. The residue was then dissolved in 1 ml hexane and analysed by gas chromatography (GC) with ion-trap mass spectrometric (IT-MS) detection in full scan acquisition mode. Following identification of residues, quantification was carried out by external calibration, either with electron capture detection (ECD), nitrogen—phosphorus detection (NPD) or IT-MS.

LC procedure for polar pesticides (Edder et al. 2005; Ortelli and Edder 2005; Ortelli et al. 2004, 2006)

After pH adjustment to 6.5–7.0, polar pesticides, such as carbamates, benzimidazoles and triazoles, were extracted from samples with 40 ml ethyl acetate and shaken mechanically for 20 min. After centrifugation at 2700 rpm for 5 min, 5 ml of supernatant was collected and evaporated to dryness without additional clean-up. The residue was then dissolved with 1 ml water/ methanol (50:50, v/v). Analyses were performed by liquid chromatography (LC) coupled to electrospray ionization and tandem mass spectrometry (ESI—MS/MS) in MRM mode. The reported multi-residues method was improved and permitted the determination of 140 pesticides commonly used in crop protection.

Analyses utilized 50 g (50 ml) of homogenized sample for the GC procedure and 20 g (20 ml) for the LC procedure.

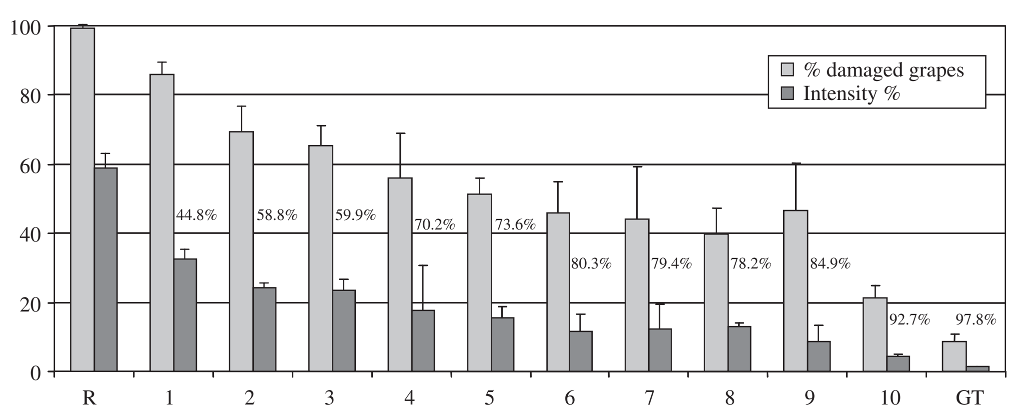
Of the fungicides applied during anti-*Botrytis* treatments, boscalid, fenhexamide, cyprodinil, fludioxonil, pyrimethanil, spiroxamin and mepanipyrim were analysed by the LC—MS/MS procedure and folpet, phtalimid, trifloxystrobin and dichlofluanid by GC.

Quantification was carried out by external calibration, and accuracy was checked by quality control samples (blank samples spiked with known pesticides concentrations). All results are the mean of two determinations. Based on EU Directive 2002/657/EC [19], an in-house validation procedure was conducted to determine method performance. According to the validation results, at least 10% of uncertainty should be taken into consideration on all measured concentrations.

Results and discussion

Efficiency of control strategies against Botrytis

In 2006, weather conditions were highly favourable for grey mould, particularly in August, with regular rainfall and temperatures approximately 2°C below the seasonal average (mean 16.8°C over August), allowing latent infection to progress without external symptoms. In September, hot weather (mean temperature 3°C above seasonal average over September) with high humidity was responsible for the appearance of the first visible rot. At the beginning of October, the week before harvest, heavy rainfall caused a very rapid spread of *Botrytis*, especially on sensitive grape cultivars such as Gamay. Figure 1 shows the efficiency of the various anti-*Botrytis* strategies on the Gamay and Gamaret cultivars expressed as % of diseased bunches and disease intensity. Assays R and 1–10 of this study were carried out on Gamay, which is very sensitive to *Botrytis cinerea*. One assay was performed on Gamaret (GT), which is more resistant to *Botrytis* and does not require specific treatment. Bars represent mean values of four replicates with standard deviations (vertical lines); the mean percentage efficiency, compared to unsprayed plots, is indicated as a number. At harvest, 100% of the untreated bunches in the control plot displayed grey mould with a mean intensity of ~60%. Under this extremely high disease pressure, important differences were observed between the strategies. The results confirm the importance of treatment before bunch closure, as shown by the single application of fludioxonil + cyprodinil (plot 6, efficiency >80%) compare to the same application at véraison (plot 3, efficiency 60%). The overall best control (plot 10, >90% efficiency) was obtained with fludioxonil + cyprodinil at bunch closure, followed by a second treatment at véraison with the broad-range fungicide trifloxystrobin mixed with dichlofluanide. Only one plot was treated previously at the end of bloom, known to be the time of first infection for *B. cinerea*, which remains latent in the berry receptacle until véraison (Viret et al. 2004). The application of fenhexamide at bloom, followed by fludioxonil + cyprodinil resulted in an efficiency of 85%, indicating that application of specific products at véraison (mid-August for the North hemisphere) is not always necessary. This can be explained by the saprophytic character of *Botrytis*, knowing that the natural defence mechanisms, concentrated in the cells of the berry skins, are no longer active after véraison (Pezet et al. 2004). Moreover, penetration of the active ingredients after bunch closure is nearly impossible for densely bunched grape cultivars. The applied fungicides act only at the bunch periphery, although latent infection spreads generally from the inner bunch structure. When the same active ingredients are applied at bunch closure and véraison (plot 5), efficiency reached 73%. A comparison of plot 7 and 10 indicates the importance of the active ingredients in treatment at



R = Gamay untreated control.
GT = Gamaret type of wine.
% values correspond to decrease of intensity compared to untreated control.

Figure 1. Efficiency of various anti-*Botrytis* fungicide treatment strategies on the Gamay and Gamaret (GT) cultivars expressed as % of diseased bunches and disease intensity. Bars represent mean values of four replicates with standard deviations (vertical lines). Mean % efficiency, compared to unsprayed plot, is indicated as a number. R = untreated control, number of assays, as in Table 1.

bunch closure. The use of a specific double-active ingredient product at bunch closure gives the best result. Interestingly, in the same experimental plot, the Gamaret cultivar (Gamay × Reichensteiner of Agroscope Changins-Wädenswil), which is resistant to *B. cinerea* (Pezet et al. 2003), recorded the best overall result, with practically no infected bunches, without any specific fungicide treatment against grey mould. This shows clearly that alternatives can be found to pesticides, even under highly favourable climatic conditions for fungal pathogens.

Pesticides residues

Table 2 shows the concentration of each pesticide residue in the grapes at harvest time, in the must and in wines after the first alcoholic fermentation (first wine) and after malo-lactic fermentation (second wine) as a function of the anti-*Botrytis* strategy applied. As mentioned in Table 1, folpet was systematically applied at flowering, excepted in the case of plot 9, where fenhexamid was also used. Folpet and its main metabolite, phtalimid, were analysed and quantified by GC with measured concentrations in grapes of between 0.9 and 6.8 $mg\,kg^{-1}$. However, these two compounds were not detected in wines, probably being degraded during the fermentation processes.

Residues of spiroxamine, another fungicide systematically used to control powdery mildew, ranged 0.015–0.11 $mg\,kg^{-1}$ in grapes and and $\sim 0.02\,mg\,l^{-1}$ in wines. Pesticide residue concentrations in wines were always below the MRLs and showed compliance when treatments were performed correctly with the registered adopted doses applied with properly calibrated spraying equipment (Viret et al. 2003, 2007; Siegfried et al. 2007). Depending to anti-*Botrytis* treatment, total pesticide residues in wines ranged between 0.37 and 0.018 $mg\,l^{-1}$ for the best case scenario. As expected, fenhexamid, cyprodinil and fludioxonil were very stable on grapes and resistant to the wine-making process. If applied on grapes, fenhexamid was found in the wine, whatever the period of treatment (bloom, bunch closure or véraison), with an increased residue when spraying occurred close to harvest. Indeed, a concentration effect during grape-pressing could occur and measurement uncertainty (~20%) needs to be considered. Final levels of fenhexamid up to 0.23 $mg\,l^{-1}$ were found in the wine.

Similar observations were recorded for boscalid with final concentrations in wine of up to 0.3 $mg\,l^{-1}$, corresponding to ~75% of grape contamination. Furthermore, the efficiency obtained in plot 1 was only ~45%.

Cyprodinil and fludioxonil, which were used in a mixed formulation, were detected in grapes at concentrations ranging between 0.1 and 0.3 $mg\,kg^{-1}$. Due to their good aqueous solubility, cyprodinil and, in particular, fludioxonil were also found in musts. Fludioxonil appeared to be very easily transferred in musts, with measured concentrations corresponding up to 100% of those observed in grapes. However, fludioxonil residues were reduced significantly during the alcoholic and malo-lactic fermentations, resulting in low levels of contamination in the final product (~0.03 $mg\,l^{-1}$). During pressing, cyprodinil transference from the grapes to the must appears to be less efficient, with concentrations in musts of only 25–40% of the grape contamination. However, the fermentation processes did not reduce cyprodinil residues, as concentrations were almost the same in musts and wines.

Pyrimethanil was analysed in grapes only and in the second wine fermentation. According to Fernandez et al. (2005), 50% of the contamination observed in grapes was found in the final wine (0.053 $mg\,l^{-1}$). Pyrimethanil was relatively stable on grapes, should be partially transferred to the must and also produce residues in wine.

Dichlofluanid, mepanipyrim and trifloxystrobin residues decreased significantly during pressing and disappeared almost completely from the wine during the alcoholic fermentation. According to this result, it can be assumed these three substances provide the best solution to avoiding residues in wine.

A comparison of anti-*Botrytis* efficiency and residues found in the final product leads to the following points. First, using very efficient, active substance such as fenhexamid or cyprodinil/fludioxonil, two treatments at flowering and bunch closure gives good protection against grey mould. In this case, a third fungicide application at colour change was not really necessary as it led only to a small increase in efficiency and higher residue levels in wine. Secondly, some strategies using less stable pesticides, such as dichlofluanid/trifloxystrobin, also displayed good efficiency against *Botrytis* and less residues in wine. Therefore, this approach is a good compromise. Since 2007 dichlofluanid and tolylfluanide are no longer permitted on grapevines in Switzerland as, in the presence of ozone, carcinogenic metabolites such as dimethylnitrosamin can be produced. Ozone being used for the treatment of groundwater, the risk of combination with dichlo- or tolylfluanide was considered sufficiently high to halt the use of these active ingredients.

The study was carried out on the Gamay grape cultivar, which is sensitive to grey mould. Other cultivars, such as Gamaret, are more resistant to *Botrytis* and do not require the use of specific fungicides, as shown in Figure 1. A reduction in pesticides treatments and, therefore, residues in wine is also possible by promoting, as far as possible, the culture of resistant vines and by differentiating anti-*Botrytis* treatments according to vine type.

Table 2. Fungicide concentrations in grapes at harvest and in the corresponding must and wines for each treatment strategy.

Assay no.	Pesticide residue	Grape [mg/kg]	Must	First wine [$mg\,l^{-1}$]	Second wine [$mg\,l^{-1}$]
1	Folpet	1.5	0.030	<0.01	<0.01
	Phtalimid	0.19	0.035	<0.01	<0.01
	Boscalid	0.41	1.16	0.32	0.30
	Pyrimethanil	0.14	Not measured	Not measured	0.053
	Total residue	2.3	1.22	0.32	0.35
2	Folpet	1.8	0.035	<0.01	<0.01
	Phtalimid	0.19	0.24	<0.01	<0.01
	Dichlofluanid	0.83	0.12	<0.01	<0.01
	Trifloxystrobin	0.22	0.061	<0.01	<0.01
	Total residue	3.1	0.24	–	–
3	Folpet	0.84	0.049	<0.01	<0.01
	Phtalimid	1.8	0.030	<0.01	<0.01
	Fludioxonil	0.31	0.47	0.083	0.029
	Cyprodinil	0.23	0.074	0.051	0.046
	Total residue	3.1	0.63	0.13	0.075
4	Folpet	1.7	0.039	<0.01	<0.01
	Phtalimid	0.44	0.020	<0.01	<0.01
	Trifloxystrobin	0.022	0.017	<0.01	<0.01
	Total residue	2.2	0.87	–	–
5	Folpet	2.6	0.025	<0.01	<0.01
	Phtalimid	0.27	0.026	<0.01	<0.01
	Fludioxonil	0.31	0.37	0.089	0.042
	Cyprodinil	0.26	0.068	0.044	0.046
	Fenhexamide	0.18	0.2	0.14	0.15
	Total residue	3.6	0.68	0.27	0.24
6	Folpet	1.5	0.047	<0.01	<0.01
	Phtalimid	0.41	0.022	<0.01	<0.01
	Cyprodinil	0.27	0.068	0.050	0.045
	Fludioxonil	0.19	0.21	0.049	0.037
	Total residue	2.4	0.35	0.099	0.092
7	Folpet	1.4	0.088	<0.01	<0.01
	Phtalimid	0.31	0.031	<0.01	<0.01
	Dichlofluanid	0.21	0.1	<0.01	<0.01
	Trifloxystrobin	0.09	0.065	0.007	0.006
	Fenhexamide	0.071	0.20	0.13	0.14
	Total residue	2.1	0.48	0.14	0.15
8	Folpet	0.24	0.032	<0.01	<0.01
	Phtalimid	0.55	0.016	<0.01	<0.01
	Fenhexamide	0.25	0.29	0.20	0.23
	Cyprodinil	0.19	0.048	0.035	0.037
	Fludioxonil	0.12	0.20	0.031	0.025
	Total residue	1.4	0.59	0.27	0.29
9	Folpet	2.1	0.031	<0.01	<0.01
	Phtalimid	4.7	0.032	<0.01	<0.01
	Cyprodinil	0.17	0.067	0.053	0.048
	Fludioxonil	0.13	0.28	0.057	0.030
	Fenhexamide	0.01	0.015	0.014	0.022
	Total residue	7.1	0.43	0.12	0.10
10	Folpet	4.6	0.10	<0.01	<0.01
	Phtalimid	0.36	0.039	<0.01	<0.01
	Dichlofluanid	1.23	0.048	<0.01	<0.01
	Fludioxonil	0.34	0.45	0.059	0.035
	Trifloxystrobin	0.32	0.051	<0.01	<0.01
	Cyprodinil	0.25	0.076	0.064	0.061
	Total residue	7.1	0.76	0.13	0.096

Conclusions

This study displayed a correlation between the efficiency of anti-*Botrytis* fungicide treatments and pesticide residues in the final product, i.e. wine. To present vine-growers with the best options for protecting their vineyards while minimizing pesticide residue levels in wine, various treatments strategies were tested. Among the 10 anti-fungal treatments, damage due to grey mould were reduce 45–93%, while individual pesticide residues were well below the MRLs. Treatment at the colour-change stage was not particularly useful and lead to higher systematic residue levels without a significant improvement in treatment efficiency. The study showed that interesting alternatives for anti-*Botrytis* treatment are available, thereby decreasing the risk of fungal resistance. The study was carried out on the Gamay variety, which is sensitive to *B. cinerea*. Other types of vines, such as Gamaret, are more resistant to *Botrytis* and do not require specific treatments. Follow-up studies should consider other cultivars with various sensitivities to *Botrytis*. This would allow treatment differentiation according to vine type, limiting the use pesticides in certain cases and considering the current year's climatic conditions.

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