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Review

Benefits and problems of fungicide control of *Botrytis cinerea* in vineyards of Champagne

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Summary

There are several agrochemicals to control *Botrytis cinerea*, the causal agent for gray mold in grapes, and new ones are being developed. The fungicides used to control *Botrytis* in vineyards of the Champagne are presented together with some of their deleterious effects on non-target species. Until recently, fungicides basically belonged to three groups: the carbamates, the benzimidazoles, and the cyclic imides. Treatments with these fungicides rapidly became inefficient because of the extreme variability observed within the pathogen. In the last 10 years, new chemicals such as pyrimethanil, fludioxonil, fluazinam, and fenhexamid, all from different chemical groups became available; until now *Botrytis* displayed no resistance to these chemicals in the Champagne. The problem of residues in wine and in the environment, including their deleterious effects on non-target organisms, as well as acute and sublethal toxicity of the fungicides are discussed. Solutions to limit environmental contamination are presented.

Key words: *Botrytis cinerea*, environment, fungicide, toxicity, vineyards.

Introduction

Grey mold, a serious disease of grapevine (*Vitis vinifera* L.) is caused by the fungus *Botrytis cinerea*. In northern vineyards such as the Champagne, this fungal disease is at least as important as downy mildew caused by *Plasmopora viticola* and it outweighs another major grape disease, powdery mildew caused by *Uncinula necator*. In some years, the costs for fungicides against *B. cinerea* are equivalent to the sum of costs for all other plant protection treatments in the Champagne vineyards. *B. cinerea* attacks leaves, developing shoots, inflorescences, and young berries without major consequence except that it creates primary outbreak from which later contamination may start. Symptoms in vineyards become evident at the onset of ripening when berries are most sensitive. Subsequently, the fungus may expand rapidly if climatic conditions are adequate affecting yield and wine quality (Dubos 1999). Some preventive methods exist to control the development of the pathogen, e.g. the use of less sensitive cultivars, reduced fertilization and sodding, leaf removal for cluster ventilation, and also protection against other pathogens such as powdery mildew and berry moth (Muckensturm and Decoin 2000).

Besides preventive control methods, several agrochemicals are available to control the pathogen and new ones are probably being developed. The various fungicides have preventive and curative effects, and they have successfully supported grape growers to control the spread of the pathogen. In the Champagne several fungicides have been used to control *Botrytis* and vines receive on average 2.3 botryticide treatments per season (Panon 2001). Older fungicides are either relatively inefficient or the pathogen has developed resistance, they are now being replaced by new ones that have been developed in the last decade. In this paper *Botrytis* fungicides, their benefits and deleterious effects, are presented.

Chemical control of *B. cinerea* in Champagne

Older substances: In the sixties, two dithiocarbamate fungicides were used to control the pathogen: dichlofluanid and thiram (Figure). These fungicides have a preventive action on germinating conidia, but their exact biochemical mode of action is not known. They are believed to be multi-site fungicides, inhibiting many essential enzymes containing Cu ions or SH groups. Due to structure similarities, the mode of action of dichlofluanid was also related to that of the multi-site phtalimide fungicides such as folpet and captan (Buchenauer 1990). These fungicides are not very efficient on *B. cinerea* but no resistance is known, probably because they have a multi-site mode of action.

In the early seventies diethofencarb, a phenylcarbamate, and carbendazim, a benzimidazole became available to growers (Figure). They showed a high efficacy against the pathogen. They have a systemic effect against germinating filaments and mycelium elongation. Carbendazim and diethofencarb exert their toxicity by binding to the tubulin of sensitive fungi thereby inhibiting mitosis and cell division (Buchenauer 1990). After a wide use of these compounds,

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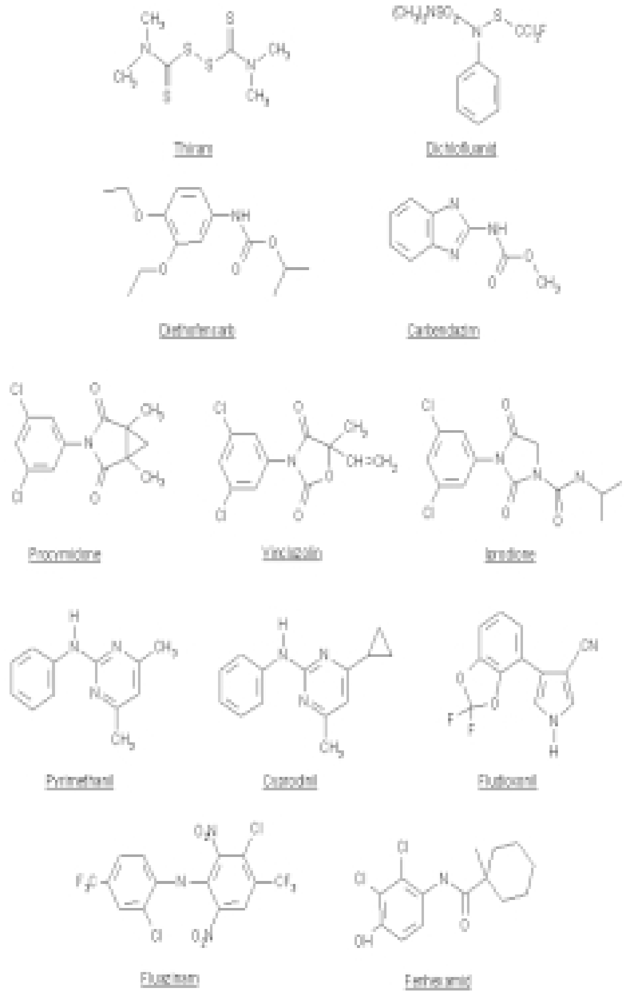

Figure: Structure of some *Botrytis cinerea* fungicides.

B. cinerea rapidly developed some resistant lines. Although their application has dramatically decreased, in 2001 in Champagne 11 % of the lines were still resistant to both compounds after a maximum of 58 % in 1991 (CIVC 2002).

The introduction of cyclic imides, e. g. procymidone, iprodione, vinclozolin (Figure), by growers and their rapid success in Champagne was certainly due to the development of resistance to benzimidazoles and phenylcarbamates in *B. cinerea*. Cyclic imides are used since the late seventies. These fungicides inhibit conidia germination and mycelium growth. Numerous physiological and biochemical effects have been reported for these fungicides. In *B. cinerea* the activity of these fungicides was correlated with lipid peroxidation (LEE *et al*. 1998), suggesting that the production of reactive oxygen species by the fungus in response to the fungicides is responsible for its toxicity. The involvement of oxygen free radicals still remains controversial, and experiments conducted with vinclozolin have shown that the toxic oxygen species do not evolve directly from the fungicide (CABRAL and CABRAL 2000). The target enzyme responsible for the direct or indirect production of reactive oxygen species is still to be discovered, possibly in the glycerol metabolism (PILLONEL and MEYER 1997). The hypothesis of a single target enzyme is favored by the fact that resistance to cyclic imides developed rapidly. In Champagne, resistant lines of *B. cinerea* appeared in the early eighties. The frequency of resistance reached a maximum of 92 % in 1981 and was down to 38 % in 1998; it is now steadily decreasing (CIVC 2001, 2002). These compounds are no longer used more than once a year; this is mainly due to the availability of new *Botrytis* fungicides.

Modern *Botrytis* fungicides: Some lines of *B. cinerea* are simultaneously resistant to carbendazim, diethofencarb and cyclic imides, lowering distinctly the grower's chance to reduce the plague. A solution to control the pathogen was found in the mid-nineties when the anilinopyrimidine fungicides became available. Pyrimethanil and cyprodinil (Figure) are the anilinopyrimidines used in Champagne. Cyprodinil was introduced only in 1999; it is only proposed in a mixture with fludioxonil, a phenylpyrrole; it is much less often used than pyrimethanil. In *B. cinerea* the anilinopyrimidines inhibit germ tube elongation and initial mycelial growth. Their biochemical primary target is believed to be cystathionine ß-lyase leading to an inhibition of methionine synthesis in the pathogen (FRITZ *et al*. 1997). The fungicides also inhibit the secretion of cell wall degrading enzymes by the pathogen (LEROUX 1996).

Some resistance of *B. cinerea* to anilinopyrimidines has already been reported (LEROUX and GREDT 1995), even with phenotypes from the Champagne vineyard (LEROUX *et al*. 1999). Nevertheless, the resistance to this class of agrochemical may virtually be neglected in Champagne (Guide Viticole 1999, CIVC 2002).

The phenylpyrrole, fludioxonil (Figure) was first used in 1995. It is a light stable derivative of pyrrolnitrin, a natural antifungal compound present in several *Pseudomonas* species (LEROUX 1996, ROSSLENBROICH and STUEBLER 2000). Fludioxonil has an effect on spore germination, germ tube elongation and also mycelium growth. Its biochemical mode of action indicates similarities with the cyclic imides. Indeed, the antagonistic action of α-tocopherol in *B. cinerea* treated with the related fungicide fenpiclonil, suggests that the action of the fungicide might be via reactive oxygen species (LEROUX 1996). It also induced an oxidative stress in algal cells (GEOFFROY 2003). Furthermore, in *Neurospora crassa* regulation of the glycerol synthesis was shown to be affected by phenylpyrroles and the primary biochemical target might be a protein kinase involved in this pathway (PILLONEL and MEYER 1997). Osmoregulation would thereby no longer be controlled in the pathogen, leading to membrane dysfunction, and possible formation of reactive oxygen species. The formation of reactive oxygen species would then appear to be a secondary mode of action.

Some resistant isolates exist (LEROUX *et al*. 1999). Some cross-resistance has even been described with cyclic imides (ROSSLENBROICH and STUEBLER 2000), this cross-resistance would not be of similar biochemical origin (STEEL 1996). Until now no resistance of practical importance was observed in Champagne (CIVC 2002).

Fluazinam (Figure), a phenylpyridinamine or 2,6-dinitroaniline from Japan was first introduced against potato blight (*Phytophtora infestans*). It was then registered for the control of *B. cinerea* in grape and became available in 1999. It has a protective contact action, little curative or systemic activity. In Champagne this compound should not be used after flowering. It inhibits spore germination, and also germ tube elongation and mycelium growth (Leroux *et al.* 1997). Biochemically, it interferes with respiration by directly uncoupling oxidative phosphorylation in the mitochondria (Leroux 1996).

In October 1999 the most recent compound, fenhexamid (Figure), a hydroxyanilide, was allowed in France. During the first season, in 2000, it became one of the major compounds used in Champagne. Fenhexamid is a strong inhibitor of germ tube elongation and mycelium growth. At high concentration it has some activity on spore germination. The integrity of the cytoplasmic membrane and/or the cell wall seems to be affected by the fungicide, since abnormal excretion is observed upon treatment of the mycelium of *B. cinerea* (Hänsler and Pontzen 1999, Rosslenbroich and Stuebler 2000). Its primary site of action has recently been found to be a 3-keto reductase in the ergosterol synthesis (Leroux *et al.* 2002). After two years of use no resistance was found in the vineyard, however, some lines resistant to other sterol inhibitors also displayed resistance to fenhexamid (Leroux *et al.* 2002).

Although the most recent fungicides are endangered by resistance, the situation is not comparable to that of the late seventies and eighties when the substances available to control *B. cinerea* were mainly derived from one family, the dicarboximides. Now growers can alternate and choose between several fungicides with different modes of action, thereby reducing the risk of resistance development.

Side effects of chemical control of *B. cinerea*

Fungicide residues in grapes and wine: A survey conducted by the French Ministry of Agriculture in the early nineties showed that almost all grape samples (80-100 % for carbendazim, iprodione, pyrimethanil, vinclozolin, and procymidone) contained residues of *Botrytis* fungicides (SDPV 1996). However, in no case the amount of residues in these samples were higher than the limits of maximum residue. The same survey also showed that most wines contained *Botrytis* fungicide residues. The transfer from grape to wine ranged between 20 and 30 % for most *Botrytis* fungicides except for carbendazim (approximately 100 %). This transfer was less important for white wines (down to 1 % for vinclozolin). The transfer of pesticide residues between treatment and harvest and their fate during wine making was recently reviewed (Cabras and Angioni 2000). In this review it is shown that among modern grape pesticides, those controlling *B. cinerea* (cyprodinil, fluazinam, fludioxonil, pyrimethanil) have the longest half- life on grapes except for fluazinam (DT$_{50}$ = 4.3 d). The longest half-life is that of pyrimethanil (57 d), and the concentration of its residues in wine was 90 % of that on grapes. In contrast, no residues of fluazinam were detected in wines. The presence of fungicides (cyprodinil, fludioxonil, pyrimethanil, and fenhexamid) in grapes did not appear to negatively influence alcoholic fermentation of wine (Cabras *et al.* 1999, 2003).

Fungicides in the environment: Water-runoff is an important phenomenon on steep slopes of the vineyards in Champagne where the mean slope is 12 %. The occurrence of pesticides in natural surface and underground water depends on its water solubility (> 30 mg·l^{-1}), the adsorption coefficient (K$_{OC}$ <500), and half-life (DT$_{50}$ > 21 d) (Hock *et al.* 1995). Because of their high DT$_{50}$ and despite their low water solubility (Tab. 1), fluazinam, cyprodinil, and the dicarboximides are potential soil and water contaminants. Combining a relatively low K$_{OC}$, a high water solubility and a relatively high DT$_{50}$, residues of pyrimethanil are potentially to be recovered although field studies have indicated low leaching to groundwater and rapid disappearance from surface water (Tomlin 2000) and

Table 1

Properties of *Botrytis* fungicides that may have an influence on residue recovery in the environment (data are adapted from Tomlin 2000, Anderson *et al.* 1999^{1}, and INRA 2003^{2})

	K$_{D}$	K$_{OC}$	Water solubility (mg·l^{-1})	K$_{OW}$ (Log P)	DT$_{50}$ Soil - field
Carbendazim		200-250	8	1.51	8-32 d
Cyprodinil		1550-4393^{2}	13	4.0	20-60 d
Dichlofluanid		500-2000^{2}	1.3	3.7	unstable
Diethofencarb	0.58-10.83^{2}	50-275^{2}	26.6	3.02	1-6 d
Fenhexamid	2.45-10.75^{1}	446-1226^{1}	20	3.51	< 1d^{1}
Fluazinam	143-820	1705-2315^{2}	1.7	3.56	33-62 d
Fludioxonil		12000-380000^{2}	1.8	4.12	10-25 d
Iprodione		373-1551	13	3	20-160 d
Procymidone			4.5	3.14	4-12 w
Pyrimethanil		265-751	121	2.84	7-54 d
Thiram		2245-24526^{2}	18	1.73	0.5 d
Vinclozolin		100-735	2.6	3	weeks

K$_{D}$ = Adsorption coefficient, K$_{OC}$ = Adsorption coefficient adjusted for the proportion of organic carbon of the soil.

K$_{OW}$ = Partition coefficient between octanol and water.

soils (Capri *et al.* 2001). In literature, herbicides are most commonly reported in runoff water, however fungicide transfer should not be neglected especially in vineyards. For example, a fungicide concentration as high as $60\ \mu g \cdot l^{-1}$ was recovered in a small river from a Beaujolais watershed (Rivenez *et al.* 1998). In this river the concentration of pesticides reached $119\ \mu g \cdot l^{-1}$. Carbendazim was reported in the Ebro delta in Spain and vinclozolin in the river Po in Italy (Readman *et al.* 1997). The same study showed that in the Ebro delta the high carbendazim concentrations (up to $200\ \mu g \cdot l^{-1}$ in rice fields and $6.5\ \mu g \cdot l^{-1}$ in drainage canals) did not result from grapevine production. In Champagne the fungicides may end up in surface water and are potential risks for aquatic flora and fauna as well. Monitoring the content of three *Botrytis* fungicides recovered in runoff water from a small watershed planted with grapes, our laboratory found procymidone, fludioxonil and pyrimethanil in the water. The highest concentration was observed for procymidone ($18\ \mu g \cdot l^{-1}$). Procymidone residues ($> 0.1\ \mu g \cdot l^{-1}$) were also reported in the Vesle river near the city of Reims in Champagne (BRGM and FREDONCA 2002). Now that several active ingredients are available to growers, the concentrations recovered in runoff water will probably decline, although the number of substances present in water may increase.

Since they may be transferred to runoff water, fungicides sprayed on soil and washed off the leaves may have some deleterious effects not only on soil organisms but also on aquatic flora and fauna.

Ecotoxicity of the fungicides: Acute toxicity: In order to be registered, agrochemicals have to be subjected to investigations on their toxicity. This information is available for the *Botrytis* fungicides used in Champagne (Tab. 2). The fungicides are not toxic to warm blooded organisms, e.g. rats and birds (quail). The response of organisms varies depending on the species. For example, dichlofluanid is most toxic to trout, whereas it is not toxic to bees or earthworms, on the contrary the most toxic fungicide to earthworms is 83 times less toxic to trout than dichlofluanid. From Tab. 2 it can be seen that the most recent fungicide (fenhexamid) seems to be the least toxic for most organisms.

However, the data in Tab. 2 only indicate the acute toxicity of fungicides; *i.e.* the effect of a single dose or exposure. It does not give any indication on sublethal effects of the compounds, nor on the interactions the fungicides (or their metabolites) may have with other agrochemicals that may be present in the environment.

Effects on plants and algae: In literature, effects of fungicides on green plants are scarce. There are some results indicating toxicity of carbendazim (mixed with propiconazole) towards pollen grain development (Pavlík and Jandurová 2000) and some effects of high concentrations of fludioxonil (1.2-30 mM) and pyrimethanil (3-75 mM) on grape carbohydrate physiology (Saladin *et al.* 2003). Other results originate from our laboratory (Verdisson *et al.* 2001, Frankart *et al.* 2001, Geoffroy 2003).

Toxicity of procymidone, pyrimethanil and fludioxonil towards *Lemna minor* is very low, IC50 were respectively > 100, 46.14, and > 100 $mg.l^{-1}$ (Verdisson *et al.* 2001). Nevertheless, the presence of these pesticides in water was not totally innocuous for this species. Indeed, some biochemical or physiological parameters may be altered. Sublethal concentrations of procymidone and pyrimethanil were able to synergize the inhibitory effects of other water contaminants such as copper and flumioxazin (Frankart *et al.* 2001).

The toxicity of *Botrytis* fungicides to another aquatic organism, the unicellular green alga *Scenedesmus* is shown in Tab. 2. *Scenedesmus obliquus* was also relatively insensi-

Table 2

Acute toxicity of *Botrytis* fungicides to various organisms
(data adapted from Tomlin 2000; Ecker and Pflüger 1999[1]; Verdisson *et al.* 2001[2] and INRA 2003[3])

	Rat Acute oral LD50 $mg \cdot kg^{-1}$	Quail Acute oral LD50 $mg \cdot kg^{-1}$	Trout LC50 (96h) $mg \cdot l^{-1}$	Bees LD50 (contact) $\mu g \cdot bee^{-1}$	Earthworm LC50 (2w) $mg \cdot kg\ soil^{-1}$	Daphnia LC50 (48h) $mg \cdot l^{-1}$	*Scenedesmus subspicatus* EC50 (72h) $mg \cdot l^{-1}$	*Selenastrum capricornutum* EC50 (120h) $mg \cdot l^{-1}$
Carbendazim	>15000	>5826	0.83	>50	6 (4w)	0.13-0.22	419	1.3 (72h)
Cyprodinil	>2000	>2000	0.98-2.41	>101	192	0.033-0.1	0.75	
Dichlofluanid	>5000	>5000	0.01	not toxic	>890 (4w)	>1.8	16	
Diethofencarb	>5000	>2250	>18	20	>1000[3]	>10 (3h)		
Fenhexamid	>4775[1]	>2000[1]	1.24[1]	>188[1]	>1000[1]	>18.8[1]	>26.1[1]	8.43[1]
Fluazinam	>5000	1782	0.11	>200	>1000 (4w)	0.22		> 0.2 (96h)[3]
Fludioxonil	>5000	>2000	0.5	>101	>1000	1.1	0.93	0.092
Iprodione	>2000	>2000	4.1	>400	>1000 (4w)	0.25		1.9
Procymidone	>6800	>6600[3]	7.2	not toxic			4.56 (*S. obliquus*)[2]	
Pyrimethanil	>4150	>2000	10.6	>100	625	2.9	23.14 (*S. obliquus*)[2]	
Thiram	2600	>3950	0.128	73.7	540	0.21		
Vinclozolin	>15000	>2510	22-32	not toxic	not toxic	4		

tive to fludioxonil (IC50 = 4.54 mg·l^{-1}, VERDISSON *et al.* 2001), however its catalase activity was stimulated (about 50 %) after incubation (12 h) in the presence of 1 mg·l^{-1} of the fungicide suggesting an oxidative stress (GEOFFROY 2003). Yet, these concentrations are by far higher than the residue concentrations that may be present in surface water.

In addition to their direct toxic lethal or sublethal effect on plants, pesticides may have complex effects on ecosystems and thereby indirect effects on plants. In freshwater microcosm, for example, carbendazim was found to increase the abundance of phytoplankton and *Elodea nuttallii* whereas it decreased the abundance of the periphytic algae (VAN DEN BRINCK *et al.* 2000). In this study the fungicide decreased the population of zooplankton grazing on the phytoplankton, it also removed some pathogen organism from *E. nuttallii* whereas some snail species that were favored grazed on the periphyton.

Effects on beneficial microorganisms: Fungicides sprayed on the soil or washed off from the leaves may alter the soil microflora and fertility. Although it is not relevant for vineyard, thiram was found to inhibit nodulation by *Rhizobium* (ANDRES *et al.* 1998). Carbendazim reduced phosphorus uptake by the hyphae of arbuscular mycorrhiza of wheat (SCHWEIGER and JAKOBSEN 1999).

Growth of some lines of *Trichoderma* which have been shown to be biological antagonists to *B. cinerea* (ELAD 1996) may be inhibited in the presence of fludioxonil (HOWELL 1999).

Effects on invertebrates: Aquatic invertebrates appear more sensitive to *Botrytis* fungicides than terrestrial invertebrates. For various species from different classes the toxicity of carbendazim shows some LC50 (96 h) values between 55 and 821 µg·l^{-1}, the concentrations at which the same organisms no longer responded to stimuli was even lower (VAN WIJNGAARDEN *et al.* 1998).

Some of the fungicides were described to have some toxicity towards beneficial grapevine arthropods such as phytoseids. Thiram, vinclozolin, iprodione, procymidone are slightly toxic (death rate between 21 and 40 %), and carbendazim is toxic (death rate between 41 and 61 %) (SPV-UIPP 1998).

Effects on vertebrates: Acute toxicity is presented for some vertebrates in Tab. 2. Although vinclozolin and procymidone are not toxic to rats (Tab. 2), it has been described that these fungicides act as antiandrogen and are capable to alter sexual differentiation in this animal (OSTBY *et al.* 1999). Furthermore, vinclozolin may delay puberty in male rats (MONOSSON *et al.* 1999), having therefore an influence on the offspring. Similarly carbendazim, which is nontoxic to most mammalian organs, was shown to be toxic to the male reproductive system leading to sterility (HESS and NAKAI 2000). In *Pimephales promelas*, a teleost fish, a 21-d-exposure to 700 µg·l^{-1} vinclozolin induced a marked reduction in female gonadal condition which did not appear to affect reproduction when the fish were returned to clean water for 4-6 months (MAKYNEN *et al.* 2000).

Histological investigations in *Rutilus rutilus* showed that procymidone induced severe damage to the liver: after 14 d 40 % of the hepatocytes were lyzed. Detoxification metabolism (EROD activity) was stimulated after 4 d with 0.4 mg·l^{-1} and decreased thereafter; the activity of Cyt P450 and Cyt b5 was reduced under the same conditions. Procymidone concentrations < 10 µg·g^{-1} induced in the same livers a drastic increase of detoxification metabolism while slightly higher concentrations induced a strong decrease of theses activities (PARIS-PALACIOS *et al.* 2001). This fungicide also induced oxidative stress and metallothionein accumulation in *R. rutilus* (PARIS-PALACIOS *et al.* 2003). Similarly, sublethal concentrations of fludioxonil also induced liver damage in *R. rutilus*, these damages were accompanied by an increase in catalase activity in the liver (BIAGIANTI-RISBOURG *et al.* 2001).

Solutions for Champagne: Integrated management is developing in the vineyards of Champagne reducing cost due to lower rates of fungicide application and increasing biological diversity (DESCOTES *et al.* 1997). Preventive methods such as lower amounts of nitrogen fertilization and *Botrytis* tolerant cultivars, are advised by extension agents and technical services even if these methods may reduce yield (PANON 2000). Treatments should only be performed in sensitive areas of the vineyards. In case treatments are necessary a succession of three fungicides belonging to three different chemical groups are advised: (1) at stage A after flowering, in order to avoid early contamination, (2) at stage B before berries have reached their maximum size so that all conidia within the cluster are reached and destroyed, and (3) at the onset of ripening (veraison) to avoid late contamination (CIVC 2001). The majority (68 %) of the growers follow this recommendation and perform three fungicide treatments per year (PANON 2000). Experimentally, *Botrytis* fungicides were applied according to mathematical models with equivalent protection (DESCOTES *et al.* 1997).

Growers are advised to regularly check their sprayers for adequate dosage of the fungicides. Some preventive methods are also used to avoid accidental tank wash and acute accidental pollution.

In order to avoid environmental water contamination, grass should grow between rows and around the fields. Runoff water is frequently conducted into decantation basins in which the pesticides are allowed to undergo degradation before the water is directed to the river via another basin (ARNOULT 1999).

New preventive methods are being investigated to control *B. cinerea* in grape. Molecular biological techniques are proposed for genetic improvement of vines, *i.e.* to produce *Botrytis* resistant vines. Another solution may be to stimulate defense mechanisms in grape, e.g. with laminarin, a compound extracted from algae (Aziz *et al.* 2003). This has already been tested in the field since a seaweed extract is available to growers (JEANDET *et al.* 1999). However, this stimulation of defense does not generate enough reliable protection yet. Results from our laboratory showed an inhibition of *Botrytis cinerea* development by chitosan (AMBORABÉ *et al.* 2003), suggesting that this fertilizer might also be incorporated into integrated protection programs against the pathogen. Until this research offers sustainable results to growers, fungicides will continue to be used with their advantages and drawbacks.

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