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Effects of fludioxonil on *Botrytis cinerea* and on grapevine defence response

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Summary. Botrytis bunch rot of grapes is mainly controlled by applying fungicides at three crop stages: the end of flowering (BBCH 68), bunch closure (BBCH 77) and the beginning of veraison (BBCH 81). The phenylpyrroles derivative fludioxonil is among the most effective fungicides registered to control *Botrytis cinerea*. Its effectiveness was investigated in relation to spray timing, fungicide resistance and defence responses of grapevine. Frequencies of *B. cinerea* strains which were resistant to fungicides were evaluated at harvest. The frequencies of resistant phenotypes were similar in all treatments except for a class of multidrug resistant strains (MDR 1) whose frequency increased after fludioxonil applications. None of the treatments tested induced defence responses in flowers/berries after fungicide application, suggesting that fludioxonil effectiveness was not related to a stimulation of plant defence processes. The standard program of three fungicide applications provided the best control of *B. cinerea* in the Champagne region in comparison with a single treatment of fludioxonil at any of the crop stages tested.

Key words: fungicide resistance, grey mould, treatment efficacy.

Introduction

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is a widespread fungal pathogen, responsible for the grey mould disease, and Botrytis bunch rot of grapevine causes severe damage in vineyards around the world (Bulit tableand Dubos, 1988). In the vineyards in Champagne, France, *B. cinerea* is especially feared by grape growers because of considerable economic losses related to this pathogen. Depending on the year, incidence of *Botrytis* bunch rot can reach 15–25% of bunches infected (Panon *et al*., 2006). In addition, wines prepared from infected grapes usually exhibit organoleptic defaults, such as oxidation of the colour or the occurrence of typical aromatic notes ("mouldy", "rotten") which are not appreciated by consumers, and alteration of foaming properties (Bocquet *et al*., 1995; Marchal *et al*., 2001; Cilindre *et al*., 2007, 2008).

In association with cultural methods of disease control, use of chemical fungicides against *B. cinerea* remains the main way to reduce the incidence and severity of bunch rot. Several classes of fungicides are available (Leroux *et al*., 2002). A standard fungicide application program consisting of three preventive applications of fungicide was recommended until 2006 in the Champagne region: at the end of flowering (BBCH 68), at bunch closure (BBCH 77) and at the beginning of berry ripening (veraison, BBCH 81) (Meier *et al*, 2001). In most cases, the fungicides consisted of fenhexamid (at BBCH 68), fludioxonil (at BBCH 77) and pyrimethanil (at BBCH 81). Fludioxonil is among the most effective fungicides registered for con-

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trol of *B. cinerea* since it inhibits spore germination, germ-tube elongation and mycelium growth (Hänβler and Pontzen, 1999). Investigations on the mode of action of this chemical have suggested that it increases the glycerol content in the fungus, leading to perturbation of the osmoregulation potential (Pillonel and Meyer, 1997; Liu *et al*., 2008).

The chemical control of Botrytis bunch rot is impeded in vineyards by the development of resistant strains of *B. cinerea* to several classes of fungicides. Shortly after the introduction of benzimidazoles and dicarboximides, several cases of resistance were recorded in European vineyards (Leroux *et al*., 1999, 2002). Natural resistance to fenhexamid (phenotype Hyd R1 and Hyd R2) was detected in populations of *B. cinerea* in many vineyards, even before this fungicide was introduced for crop protection (Suty *et al*., 1999). No resistance was detected to fludioxonil in French vineyards, whereas strains of other fungi (e.g. *Stemphylium vesicarium* [Alberoni *et al*., 2010] and *Alternaria brassicicola* [Avenot *et al*., 2005]) exhibiting resistance to this fungicide were detected. In some *B. cinerea* laboratory mutants, cross-resistance was observed between phenylpyrroles (e.g. fludioxonil) and dicarboximides, but this was never detected in field isolates (Leroux, 2004; Liu *et al*., 2008). In addition to the specific resistance, multi-resistant strains of *B. cinerea* were detected in French vineyards, and especially in the Champagne vineyards, in the late 1990s. These strains differ from multiple resistant ones which accumulate various target alterations. On the contrary, the resistance mechanism is monogenic and was related to multidrug resistance (MDR), as had been observed for human pathogens. MDR strains are cross-resistant to a variety of fungicides belonging to different chemical families. This kind of resistance is not determined by target gene alterations but rather by over-expression of drug transporters located in fungal cell membranes (Kretschmer *et al*., 2009). The development of these resistant strains may influence fungicide effectiveness if they propagate in field populations at significant frequencies.

In addition to their toxicity against pathogens, some fungicides can indirectly act by changing plant physiology (Prudet, 1994) or stimulating plant defence responses (Garcia *et al*., 2003). In grapevine, inducible defence mechanisms were characterized in berries following *B. cinerea* infection. They consist in (i) induction of gene expression encoding pathogenesis-related (PR) proteins (Bézier *et al*., 2002), (ii) enhancement of chitinase and glucanase activity (Derckel *et al*., 1998), and (iii) increase of stilbene phytoalexins formed *via* the phenylpropanoid/polymalonate pathway (Jeandet *et al*., 1995). If fludioxonil treatment may be involved in the activation of these defence mechanisms to resist *B. cinerea* infection, the fungicide may increase its effectiveness against *B. cinerea* development. Although defence responses following fludioxonil application have been studied in grapevine leaves (Petit *et al.,* 2009a), there is no information available on effects in flowers or berries, although fungicide spraying against *B. cinerea* is essentially directed to the grapevine reproductive organs.

Fludioxonil was applied at different grapevine growth stages in the Champagne region of France during 4 years. Our objective was to examine fludioxonil effectiveness in relation to spray timing, fungicide resistance and defence responses. Therefore, we evaluated consequences of fludioxonil treatment on the frequency of *B. cinerea* strains which were resistant to fungicides. Defence was also quantified in grapevine reproductive organs following fludioxonil application, focusing on (i) expression of various genes coding for PR proteins and phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid/polymalonate pathway; and (ii) chitinase activity.

Materials and methods

Field trials and experimental design

Experiments were conducted in an experimental vineyard located in Loisy-en-Brie, in the Champagne region (France). This vineyard has a history of severe bunch rot every year. Grapevines (*Vitis vinifera* L. cv. Pinot Meunier), grafted on 41B rootstock and trained according to the Chablis method, were planted in 1986.

All field experiments were conducted using formulated products. Fludioxonil was formulated as the commercial fungicide Geoxe (50% a.i.; Bayer) and was applied at 1 kg ha^{-1}, at the BBCH stages 68, 77 or 81. Each treatment applied individually was compared to the standard program, which consisted of three applications of fungicide:

fenhexamid (50% a.i. Teldor-Syngenta; at BBCH 68), fludioxonil (at BBCH 77) and pyrimethanil (at BBCH 81). Fludioxonil was used at a rate of 1 kg ha^{-1} while pyrimethanil (Scala-BASF, 400 g l^{-1} a.i.) was used at a rate of 2.5 L ha^{-1}. Fenhexamid was applied at 1.5 kg ha^{-1}. Fungicides were sprayed on both sides of vines with a hand-operated backpack sprayer (250 L ha^{-1}). Non-sprayed grapevines were used as controls. Individual treatment plots (control, standard and three fludioxonil treatments) were arranged in a randomized complete block design with four replications. Each replication consisted of at least twelve grapevines. Each treated row was bordered by two unsprayed buffer rows to minimize drift of fungicide from outside the trial.

Disease assessment

During each harvest from 2002 to 2007, bunch rot infection was evaluated on two clusters per grapevine *i.e.* about 25 clusters per replication and a total of 100 clusters. Incidence of bunch rot was calculated as the percentage of infected clusters (showing at least one rotten berry with typical symptoms). In addition, disease severity was assessed as the percentage of symptomatic berries (area rotten and/or sporulating with *Botrytis*) in each cluster.

Characterization of *B. cinerea* populations

Field populations of *B. cinerea* were isolated from diseased berries at harvest. A minimum of 20 infected berries with sporulating *B. cinerea* per treatment plot were randomly collected. Berries were suspended in 15 ml of sterile water, without surfactant, and vigorously shaken. The phenotypes were characterized according to Leroux *et al*. (1999): the bulk conidium suspension was adjusted to 300,000 conidia mL^{-1} with the aid of a haemocytometer and then 300 μL were used directly to inoculate 55 mm diameter Petri dishes containing agar medium amended with doses of various fungicides previously shown to discriminate the various phenotypes (Leroux *et al*., 1999). Microscopic observations at ×100 magnification of a minimum of 100 conidia per treatment were carried out after 24 h and 48 h to determine the proportion of germinated conidia with long germ tubes (representing at least 50% of the length of conidia in experimental control, i.e. on-amended medium. This was to evaluate the frequency of resistance to anilinopyrimidines (Ani R1), benzimidazoles and phenylcarbamates (Ben R1 and Ben R2), dicarboximides (Imi R1), hydroxyanilides (Hyd R1 and Hyd R3) as well as multidrug resistant (MDR) strains (phenotypes MDR 1 and MDR 2, distinguished respectively

Table 1. Phenotypes of sensitivity towards several fungicides described in field populations of *B. cinerea*; analysis according to germ tube elongation. Phenotypes were classified according to the resistance levels (RL) calculated for the various fungicides (RL=LC_{50} resistant strain/LC_{50} sensitive strain); HS, hypersensitive (RL<0.5); S, sensitive (0.5<RL<2); LR, low resistance (2<RL<10); MR, moderate resistance (10<RL<25); HR, high resistance (RL>25).

Fungicide families	Phenotypes of sensitivity							
	Ben R1	Ben R2	Ani R1	Imi R1	Hyd R1	Hyd R3	MDR 1	MDR 2
Benzimidazoles	HR	HR	/	/	/	/	LR	LR
Phenylcarbamates	HS	HR	/	/	/	/	LR	LR
Dicarboximides	/	/	/	MR	/	/	LR	LR
Phenylpyrroles	/	/	/	S	/	/	MR	S/LR
Anilinopyrimidines	/	/	MR/HR	/	/	/	LR/MR	LR/MR
Hydroxyanilides	/	/	/	/	LR	MR/HR	S/LR	LR/MR

by their higher resistance to fludioxonil and fenhexamid) (Leroux *et al*., 1999) (Table 1).

Grapevine defense responses

RNA extraction and Real-time PCR analysis

In 2006 and 2007, one apparently non-infected inflorescence/cluster per plant from eight plants treated with fludioxonil or untreated was collected 24 h after fungicide spraying at stages BBCH 68, 77 or 81. They were immediately frozen in liquid nitrogen then stored at -80°C. Flowers/berries were separated from each bunch stem (Jackson and Coombe, 1995) and were then ground in liquid nitrogen to a fine powder. For flowers, a 100 mg aliquot of powder was used for total RNA extraction and homogenized in extraction buffer (Plant Purification RNA Reagent, Invitrogen), according to the manufacturer's instructions. For berries, total RNA was extracted according to the method of Davies and Robinson (1996). Each RNA pellet was resuspended in 20 μL of RNase-free water and quantified by absorbance at 260 nm. RNA was stored at -80°C until use for RT-PCR.

A 150 ng aliquot of total RNA was reverse-transcribed using M-MLV reverse-transcriptase (Invitrogen) according to the manufacturer's protocol. PCR conditions were as described in Bézier *et al*. (2002). The reaction was carried out in duplicate in a GeneAmp 5700 sequence detection system (Applied Biosystems) using the following thermal profile for 40 cycles: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension). The copy number for each sample was calculated according to Petit *et al*. (2009a). The induction factor following fludioxonil treatment was calculated: the results were normalized using the gene EF1α as an endogenous control and data were expressed as -fold change relative to the control samples (untreated flowers/berries). Expression of four defence-related genes encoding class IV chitinase (*Chi4C*), β-1,3-glucanase (*GLUC*), class 6 pathogenesis-related protein (*PR6*) and phenylalanine ammonia-lyase (*PAL*) were tracked (Table 2).

Chitinase extraction and activity

As described above for RNA extraction, in 2006 and 2007, one inflorescence/cluster per plant from eight plants treated with fludioxonil or untreated was collected 24 h after fungicide spraying at stages BBCH 68, 77 or 81. Flowers/berries were separated from each bunch stem. Protein extraction was performed according to Petit *et al*. (2009a)

Table 2. Defence-related genes analyzed by real-time RT-PCR. The mRNA copy number of each sample was calculated from the standard curve using its Ct value and corrected by normalization against EF1α mRNA (Terrier *et al*., 2005).

Gene	Encoding	Primer sequence	Accession No.
EF1-α	Elongation factor 1-alpha	Sense 5' GAA CTG GGT GCT TGA TAG GC 3' Antisense 5' AAC CAA AAT ATC CGG AGT AAA AGA 3'	BQ799343
Chi4C	Class IV chitinase	Sense 5' TCG AAT GCG ATG GTG GAA A 3' Antisense 5' TCC CCT GTC GAA ACA CCA AG 3'	AY137377
Gluc	β-1,3-glucanase	Sense 5' TCA ATG GCT GCA ATG GTG C 3' Antisense 5' CGG TCG ATG TTG CGA GAT TTA 3'	AF239617
PR6	Class 6 pathogenesis-related protein	Sense 5' AGT TCA GGG AGA GGT TGC TG 3' Antisense 5' GCA CTA GGG TCC GTG TTT GGG TCG ACG 3'	AY156047
PAL	Phenylalalanine ammonia-lyase	Sense 5' TCC TCC CGG AAA ACA GCT G 3' Antisense 5' TCC TCC AAA TGC CTC AAA TCA 3'	X75967

and then chitinase activity was assayed using a commercial blue enzyme substrate, CM-chitin-RBV solution (Loewe Biochemica) according to Magnin-Robert *et al*. (2007). Measurements were conducted in triplicate. Results were expressed in mg min^{-1} g^{-1} fresh weight (FW).

Statistical analysis

Values of disease incidence and severity represent means of data from 2002 to 2007. Results of gene expression and chitinase activity represent means of replicates performed over 2 years. To determine whether values of control plants and plants of treatment plots were significantly different, analysis of variance (ANOVA) followed by a Student's *t* test were used. Differences at $P<0.05$ were considered as statistically significant.

Results

Effectiveness of fungicide treatments

In control plants, mean disease incidence was close to 75% and decreased to 35% when plants were treated with the standard reference program (Figure 1). Incidence decrease was not significant when fludioxonil was applied at stages BBCH 68 and 81 but declined to about 25% for fungicide applied at stage 77. Mean bunch rot severity was close to 27% in control plants and significantly reduced by 4-fold after treatment with the standard reference program (Figure 1). After fludioxonil application, severity was reduced similarly whatever the stage of application and was close to 25%.

Sensitivity to fungicides

Similar frequencies of benzimidazole (Ben R1 and Ben R2), anilinopyrimidine (Ani R1), dicarboximides (Imi R1) and hydroxyanilides-resistant strains (Hyd R1 and Hyd R3) were found from control and treated plants, whatever the stage of treatment (Table 3). A high frequency of Ben R1 strains was observed varying between 42.5 and 53.3%. Proportions of Ben R2, Ani R1, Imi R1, Hyd R1 and Hyd R3 strains were lower, varying between 0 and 20.0%.

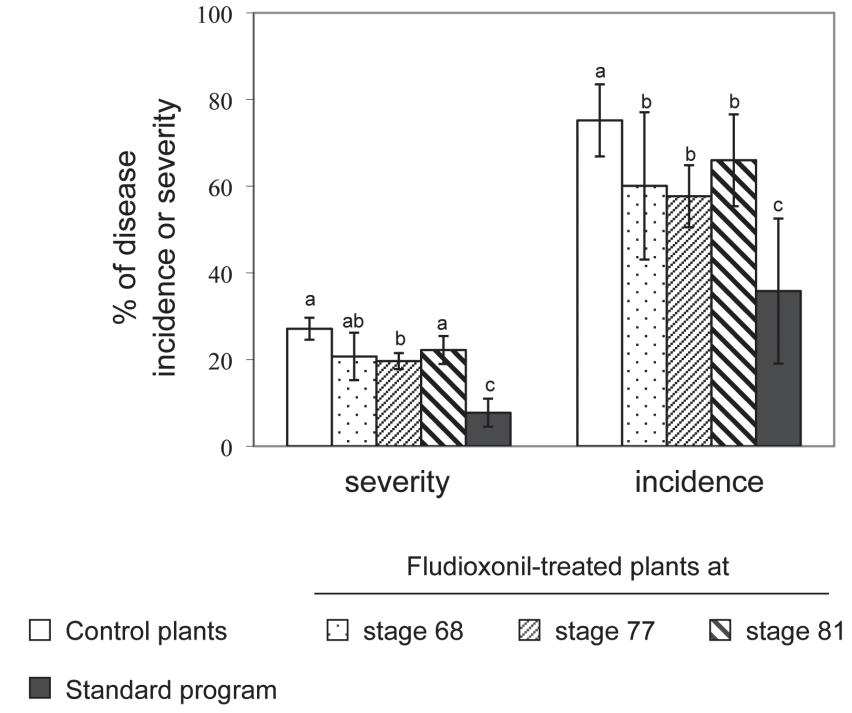

Figure 1. Mean severity (a) and incidence (b) of grey mould on grape berries at harvest, following different fungicide treatments. The effectiveness of fludioxonil is compared according to stage of application: end of flowering (BBCH stage 68), bunch closure (BBCH stage 77), or veraison (BBCH stage 81). Means with the same letter were not significantly different ($P<0.05$) as determined by the Student's *t* test.

Table 3. Percentage of resistance towards anilinopyrimidines (Ani R1), benzimidazoles and phenylcarbamates (Ben R1 and Ben R2), dicarboximides (Imi R1) or hydroxyanilides (Hyd R1 and Hyd R3) in *B. cinerea* strains, in control plants, in treated plants with fludioxonil at stages 68, 77 or 81, or with reference program. Means represent data from 2002 to 2007. No significant differences were found ($P<0.05$).

Phenotypes	Control	Fludioxonil-treated at stage			Standard program
		BBCH 68	BBCH 77	BBCH 81	
Ani R1	0.0a[a]	10.0a	0.0a	2.5a	0.0a
Ben R1	43.3a	53.3a	51.7a	42.5a	50.0a
Ben R2	2.5a	7.5a	7.5a	3.3a	2.0a
Imi R1	19.2a	5.0a	20.0a	6.7a	10.0a
Hyd R1	1.7a	1.7a	5.0a	1.0a	2.7a
Hyd R3	0.0a	0.0a	10.0a	0.0a	0.0a
MDR1	4.0a	16.0a	13.3a	20.8a	12.0a
MDR2	15.0a	23.3a	15.0a	14.2a	19.2a

[a] Values followed by the same letter are not statistically different at the 5% level by ANOVA followed by a Student's t test.

The trend observed was for a higher frequency of MDR after fludioxonil treatment compared to control plants. Similarly, an increase was noticed after the standard reference program application. MDR1 increase was at maximum with a factor 5.2 in fludioxonil-treated plants compared to control plants whereas the increase was only by a factor 1.5 for MDR2 strains.

Grapevine defence responses

No significant modification in expression of *PAL*, *LOX*, and genes encoding PR proteins (*Chi4C*, *GLUC* and *PR6*) was observed in flowers (stage BBCH 68) or berries (stages BBCH 77 and 81) following fludioxonil treatments (data not shown). Basal level of chitinase activity was 1.1 and 1.4 mg min^{-1} g^{-1} FW in control plants at stages BBCH 68 and 77, respectively (Figure 2), and was weakly higher in berries at stage BBCH 81 (1.8 mg min^{-1} g^{-1} FW). Following fludioxonil treatments, a significant 60% increase in chitinase activity was occurred only after treatment at stage BBCH 81.

Discussion

Our results showed that the standard program of three fungicide applications provided the best control of *B. cinerea* in the Champagne region in comparison with a single treatment of fludioxonil at any of the crop stages tested and in each of the years studied. Single applications of fludioxonil therefore are specifically adapted at a given vine growth stage, while a significant reduction of both disease severity and incidence was demonstrated when fenhexamid was applied at stage BBCH 68 (Petit *et al*., 2010). This indicates that application at BBCH 68 is decisive for the most effective control of grey mould disease (Nair *et al*., 1995; Jermini *et al*., 1986; Pezet and Pont, 1986). In addition, fludioxonil seems to have a greater effect on disease severity than on disease incidence, indicating that this fungicide may act by diminishing the size of fungal infection foci rather than in reducing the number of foci. Selection pressure exerted by fungicides on *B. cinerea* strains and defence responses of grapevine to fungicides were then tested to evaluate potential interactions between these factors and effectiveness of fungicide treatments.

High frequencies of Ben R1 strains were recorded in each year of this study despite the absence of selection pressure. Benzimidazoles were developed at the end of 1960's and the use of these compounds rapidly induced development of highly resistant strains, particularly in locations of intensive use such as in the Champagne region (Leroux and Clerjeau, 1985). These strains generally exhibited the mutation E198A in the gene en-

coding β-tubulin (Leroux *et al.,* 2002). These high proportions of Ben R1 strains, despite the absence of contemporary selection pressure, may indicate that the biological cost of this previous mutation is reduced (Johnson *et al*., 1994).

Conversely, the frequency of Ben R2, AniR1, Imi R1, Hyd R1 and Hyd R3 phenotypes was low or zero in most cases, including the standard reference fungicide program. The phenotype Ben R2, which is simultaneously resistant to benzimidazoles and phenylcarbamates, is generally determined by the mutation F200Y in the β-tubulin gene (Leroux *et al*., 2006). Low frequencies of this phenotype in our trials, as well as in the Champagne region, may indicate that the mutation F200Y induces a high fitness penalty. The low frequencies ImiR1 and AniR1 phenotypes confirm that their resistance was not significantly selected by the dicarboximide application and the pyrimethanil treatment applied at stage BBCH 81 respectively. Imi R1 resistance is conferred by alterations within the *Bos1* gene and the most frequent mutation is I365R/S/G (Leroux *et al*., 2002; Cui *et al*., 2004). AniR1 resistance, the mechanism of which is still unclear (Fritz *et al*., 2003), was often described as unstable, probably because of a fitness penalty (Leroux *et al*., 2004). Resistance to hydroxyanilides, low frequency of resistant strain (Hyd R1) and low specific resistance (Hyd R3) confirm that no significant efficacy loss has been recorded with fenhexamid (Leroux *et al*., 2002).

For MDR strains, MDR1 increase was greater than MDR2 in fludioxonil-treated plants. Indeed, MDR1 stains exhibited higher levels of resistance to fludioxonil (Table 1) and were probably better selected by fludioxonil, as already reported by Walker *et al*. (2006). Nevertheless, even if the highest frequencies of MDR phenotypes were recorded after fludioxonil application at stage BBCH 68, efficacy of the fungicide was below that achieved with the standard reference program, but was still better than the control and acceptable because frequency of MDR1 and associated resistant levels do not lead to great efficacy loss. In the context of this study, this could indicate that limiting the use of fungicides in French vineyards (to a maximum of one treatment by class of mode of action) is sufficient to reduce bunch rot significantly when combined with cultural control measures, and when specific resistance leading to high resistant levels is not likely to get selected.

Defence responses of grapevine reproductive organs were evaluated following fludioxonil application at the three tested vine growth stages. Several fungicides used to control bunch rot, such as benzimidazoles, strobilurins or triazoles, stimulate the increase of PR gene expression and the accumulation of PR proteins, the increase in PAL activity or the accumulation of phenolics in various crops (Siefert *et al*., 1996; Garcia *et al*., 2003; Pasquer *et al*., 2005). In grapevine, it was observed that application of dicarboximide fungicides in vineyards indirectly acted against *B. cinerea* by changing plant physiology. These fun-

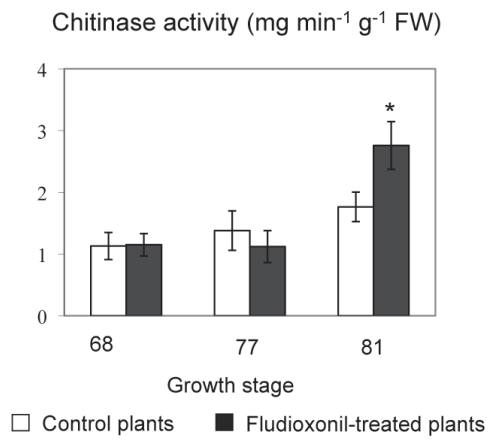

Figure 2. Mean chitinase activity in control and fludioxonil-treated flowers (BBCH stage 68) or berries (BBCH stages 77 and 81) of grapevine, 4 days after treatment. Data are means ± standard errors (n = 16). Asterisks indicate significant differences ($P<0.05$) between control and treated plants, as determined by the Student's *t* test.

gicides acted positively by conservating berry inhibition against *B. cinerea* and delayed skin destructuring. These changes led to better protection of grapevine against *B. cinerea*. Nevertheless, our results showed that no increase of the defence processes tested (changes in the level of gene expression and chitinase activity) was observed in flowers at stage BBCH 68 and in berries at stage 77 following fludioxonil application as well as following fenhexamid application (Petit *et al*., 2010). Only an increase in chitinase activity was noticed in berries at stage 81. Reduction of bunch rot was not significant after fludioxonil treatment at stage 81, suggesting that fungicide effectiveness was not related to an activation of defence responses in grapevine. The lack of response of chitinase activity in reproductive organs at flowering or bunch closure following fungicide treatment might be explained by a poor capacity to induce their defence mechanisms contrary to berries at latest stages. Indeed, although multiple defence responses were induced in berries at later stages to UV-C irradiation, no significant induction of defence responses was observed in flowers (Adrian *et al*., 2000; Bais *et al*., 2000; Petit *et al*., 2009b).

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