*Variability in the sensitivity of biotrophic grapevine pathogens (*Erysiphe necator *and* Plasmopara viticola*) to acibenzolar-S methyl and two phosphonates*

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Variability in the sensitivity of biotrophic grapevine pathogens (*Erysiphe necator* and *Plasmopara viticola*) to acibenzolar-S methyl and two phosphonates

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Abstract The antifungal properties of two phosphonates (fosetyl-Al and a fertilizer) and acibenzolar-S-methyl (ASM) were evaluated to assess their potential for protecting grapevine leaves against grapevine mildews (*Plasmopara viticola* and *Erysiphe necator*), and to determine their effects on the development of various mildew isolates, taking into account the inter- and intra-species variability of the pathogens. The phosphonates directly and significantly inhibited the growth of these pathogens. By contrast, ASM had no direct effect on spore production and growth of *P. viticola* and of *E. necator* at 1.9 mM. Applied before inoculation, the mean EC_{50} of ASM was 0.50±0.04 mM and 1.00± 0.07 mM for downy and powdery mildew isolates, respectively. The EC_{50} of the fosetyl-aluminium (FOS) was 0.50±0.02 mM for downy mildew and the EC_{50} for powdery mildew varied depending on the genetic group under consideration (0.89±0.32 mM for group B 3.30± 0.46 mM for group A, respectively). The EC_{50} of the potassium phosphonate fertilizer (PK_2) was 0.96± 0.45 mM for downy and 6.9±0.76 mM for powdery mildew isolates. These compounds showed differences in their efficacy depending on the variability of mildews and could be an alternative or additional method to traditional pest management in the grapevine.

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Introduction

Vineyards are subject to numerous parasitic pressures, among which obligate parasites such as powdery mildew (*E. necator* Schw.) and downy mildew (*P. viticola* Berk. & Curt) are very important grapevine diseases (*Vitis vinifera*). These two biotrophic pathogens, native to the United States, infect green vine tissues and cause significant economic loss as well as environmental damage through the repeated application of fungicides.

The biotrophic oomycete *P. viticola* (Berk. & Curt.), the causal agent of downy mildew, is a heterothallic endoparasite (Lafon and Clerjeau 1988). Its development involves intercellular mycelial growth and the differentiation of haustoria, which penetrate parenchyma cells. The genetic diversity of this oomycete in Europe is low, but the population exhibits various phenotypes and genotypes (e.g., fungicide resistance, Chen et al. 2007; Gisi et al. 2007). By contrast, *E. necator* (grapevine powdery mildew) is an ascomycete, and an ectoparasitic biotrophic fungus. Its populations are divided into two distinct genetic groups, A and B, in Europe and Australia (Délye et al. 1997a; Evans et al. 1997), that have different ecological requirements (Amrani et al. 2006; Montarry et al. 2008), but the distribution and the epidemiological

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significance of the two groups are unknown. Furthermore, group A isolates are significantly more sensitive to various fungicides than group B. Group A isolates also exhibit a higher sensitivity to sterol demethylase inhibitors (DMI), to quinone outside inhibitors (QoI) and to quinoxyfen. This may be related to the low selection pressure of fungicides exerted on these populations and may also depend on the asexual wintering of group A populations in France (Corio-Costet 2007; Dufour et al. 2011).

To prevent fungal diseases on leaves and berries numerous fungicide treatments were required (from two to ten), having consequences on environmental and human health (Margni et al. 2002). Furthermore, the strong selection pressure sometimes applied by uni-site fungicides on pathogens such as downy and powdery mildew can result in the recurring appearance of resistant populations and this has limited the success of fungicides on grapevines (e.g., QoI, DMI fungicides) (Dufour et al. 2011; Gisi et al. 2002; Corio-Costet et al. 2011; Delye et al. 1997b). As plants possess the ability to defend against pathogens, part of these defences can be induced and this will confer protection against a wide spectrum of pathogens (Walters et al. 2007). It would be interesting to integrate these properties of plant defence into future pest management strategies. Typically, this resistance can be induced by biological or chemical agents (Lyon et al. 2007) leading to a coordinated accumulation of pathogenesis-related proteins (PR), to production of phytoalexins, and to the reinforcement of plant cell walls (Garcion et al. 2007). To improve grapevine pest management, it is becoming increasingly difficult to ignore plant defence activators which could represent an important component of additional methods of pest management strategies for *E. necator and P. viticola*. Because they usually act on the plant and not directly on the pathogen (Siegrist et al. 1997), elicitors trigger a resistance phenomenon in the host plant which could be more difficult for the pathogen to get round than the toxic effect of a uni-site fungicide.

The ASM, a functional analogue of salicylic acid, is described as activating systemic resistance (SAR) (Friedrich et al. 1996) and has been shown to be effective against a broad spectrum of pathogens in various plants (Friedrich et al. 1996; Bressan and Purcell 2005; Brisset et al. 2000; Dann and Deverall 2000; Perazzolli et al. 2008; Pajot and Silué 2005). The effect of ASM on plants leads to subsequent regulation of genes involved in primary and secondary metabolism, but also to an accumulation of phenolic compounds (Brisset et al. 2000; Lawton et al. 1996; Hukkanen et al. 2008; Iriti et al. 2004; Bovie et al. 2004; Dufour et al. 2012).

Phosphonates or phosphites ($H_2PO_3^-$), a generic term used for mono or di-potassium salts of phosphoric acid (H_3PO_3), are being widely marketed either as a source of plant nutrition or as a fungicide to control oomycetes (MacDonald et al. 2001; Lobato et al. 2008). The effect of phosphonates in controlling the pathogen is determined by the phosphonate concentration and when phosphonate concentrations are low, the product interacts with the pathogen at the site of ingress to stimulate host defence enzymes (Jackson et al. 2000; Massoud et al. 2012). Their mode of action is complex and may directly or indirectly inhibit the pathogen by activating plant defence mechanisms (Smillie et al. 1989; Guest and Grant 1991; Hardy et al. 2001; Daniel and Guest 2006; Eshraghi et al. 2011; Massoud et al. 2012). Fosetyl-Al, a phosphonate derivative, is a systemic fungicide which has acropetal and basipetal mobility and is active against oomycetes (Bompeix and Saindrenan 1984; Fenn and Coffey 1985). Besides its fungicide activity, FOS is also described as activating disease resistance mechanisms, such as phytoalexin production in plants (Dercks and Creasy 1989; Guest 1984; Andreu et al. 2006). In the grapevine, it was more efficient in the post-infection period than in the pre-infection period and defences of grapevine were activated only in the presence of *P. viticola* (Lafon et al. 1977). Other phosphonates, e.g., potassium phosphonate PK_2, used as fertilizers also seemed to present an action of stimulating the natural defences of the grapevine (Soyez 2001). Indeed, crops treated with this product suggested the existence of defence stimulation with a premature presence of ethylene, a premature accumulation of lignin and phytoalexins around the site of infection. It seems that all phosphonates are able to stimulate plant defences.

However there are no data on the efficacy of these products faced with the variability of grapevine obligate pathogens and their behaviour. This point could be important, although we want to use these compounds as an additional method to the conventional fungicides used in vineyard pest management with a view to decreasing the quantity of pesticides. The use of compounds with a broad spectrum of action on different pathogens, able to stimulate plant defences

and/or to have a minor action on pathogens, would be an advantage within the framework of the reduction of pesticide use in the vineyard. The few studies existing have tended to focus on direct efficacy in the vineyard rather than on an explanation of the variability of responses obtained with these compounds in the presence of different pathogens (Campbell and Latorre 2004; Lafon et al. 1977)

The aim of the present study was to investigate and compare the efficacies of ASM and phosphonates on various isolates of the two major grapevine pathogens. The overall objectives were: (i) to assess the effect of two phosphonates (FOS and PK_2) and ASM on the development of *P. viticola* and *E. necator*, and (ii) to examine the role of pathogen diversity on their efficacy in order to optimize future applications in the vineyard. The utilization of phosphonates or ASM could be considered as a possible strategy to be included in an integrated pest management program in order to reduce the intensive use of fungicides and the phenomenon of fungicide resistance.

Materials and methods

Plant material

Grapevine plants (*V. vinifera* cv. Cabernet Sauvignon) were propagated in a greenhouse at 25 °C with a 15:9 h light: dark photoperiod. Two-month-old plants were used for the experiments, and the third and fourth leaves at the apex of plants were used for powdery mildew and downy mildew inoculations, respectively.

For powdery mildew experiments, whole adaxial surfaces of leaves were disinfected by immersion (10 min) in a solution of 5 % calcium hypochlorite solution (50 $g l^{-1}$), rinsed and dried between two sheets of sterilised filter paper. Leaf discs were obtained with a pastry-cutter 18 mm in diameter and were placed in Petri dishes containing agar (20 $g l^{-1}$) supplemented with benzimidazole (30 $mg l^{-1}$) or on Whatman's paper moistened with 2.5 ml of sterile water.

For downy mildew experiments, whole abaxial surfaces of leaves were washed, dried between two sheets of sterilised filter paper and 18 mm-wide leaf discs obtained with a pastry-cutter were placed in Petri dishes on Whatman's paper moistened with 2.5 ml of sterile water.

Pathogen materials

P. viticola

Two fungicide-sensitive isolates and four fungicide-resistant isolates from the laboratory monospore collection were used, multiplied on grapevine leaves and inoculated according to the procedure used by Corio-Costet et al. (2011) (Table 1), with a spore suspension at 5000 sporangia ml^{-1}, twenty drops of 15 μl per leaf or three drops per leaf disc, and placed in a growth chamber (mean temperature 22 °C, with 16 $h day^{-1}$ light, 350 $\mu m m^{-2} s^{-1}$) for 7 days.

E. necator

Eight powdery mildew isolates belonging to genetic group B, and 10 belonging to genetic group A, used in the assays came from the laboratory collection (Table 1). Monoconidial isolates were inoculated under sterile conditions on decontaminated grape leaves (cv. Cabernet Sauvignon) in Petri dishes as described in a previous article (Délye et al. 1998). Leaves or leaf discs were placed at the bottom of a Plexiglas settling tower and conidia were blown in at the top from sporulating leaves (1000–1500 conidia per cm^2 of leaf). Inoculated leaves or discs were placed in a growth chamber (mean temperature 22 °C, with 16 h day^{-1} light, 350 $\mu m m^{-2} s^{-1}$) for 12 days.

Effect of ASM and phosphonate concentrations on pathogen growth

The suspensions of product were prepared extemporaneously for the experiments. ASM (S-methyl benzo[1,2,3]thiadiazole-7-carbothioate, Bion®, 50WG), FOS (aluminium tris (ethyl phosphonate), Aliette®) and a potassium phosphonate (PK_2®) were dissolved in water. The effect of products on the mycelial growth of *E. necator* and *P. viticola* was assessed in laboratory, using six different concentrations of ASM, FOS and PK2 (100, 250, 500, 750, 1000 and 1500 mg l^{-1} of a.i.). ASM was so applied at 0.47, 1.18, 2.36, 3.54, 4.72 and 7.08 mM, FOS at 0.28, 0.70, 1.40, 2.11, 2.81 and 4.21 mM and PK_2 at 0.63, 1.59, 3.18, 4.78, 6.37 and 9.55 mM.

The tests were carried out by spraying dilutions with a micro-diffuser having a pressure reserve (Ecospray ®) on eight leaf discs, as described in detail

Table 1 Characteristics of *P. viticola* and *E. necator* isolates

Pathogen	Isolates	Locality of vineyard	Sampling year	Fungicide sensitivity		
				FAM[a]	IPRO[b]	MEF[c]
P. viticola	PIC 59	Bordeaux	2003	R	S	R
	MIC 128	Bordeaux	2005	R	R	S
	PAU 32	Bordeaux	2003	R	S	R
	EAU 08	Armagnac	2005	R	R	S
	FLE 01	Beaujolais	2004	S	S	S
	COU 15	Bordeaux	2004	S	S	S
				Genetic group		
E. necator	PVR 43	Languedoc-Roussillon	2003	A		
	BR 33	Pays de l'Aude	2002	A		
	LLU 70	Pyrénées-orientales	2006	A		
	LLU 41	Pyrénées-orientales	2006	A		
	LLU 55	Pyrénées-orientales	2006	A		
	PV 15	Pays de l'Aude	2006	A		
	PV 33	Pays de l'Aude	2006	A		
	PVR 38	Languedoc-Roussillon	2005	A		
	ORA 4	Languedoc-Roussillon	2003	A		
	ORA 3	Languedoc-Roussillon	2003	A		
	LAT 12	Bordeaux	2003	B		
	CC 49	Bordeaux	1999	B		
	CHL 02	Bordeaux	2004	B		
	PAL 01	Bordeaux	2003	B		
	2B 15	Pays de l'Aude	2000	B		
	PV 28	Pays de l'Aude	2006	B		
	PV 46	Pays de l'Aude	2006	B		
	PV 74	Pays de l'Aude	2006	B		

S sensitive isolate, *R* Resistant isolate

[a]Sensitivity to fungicide famoxadone

[b]Sensitivity to iprovalicarb

[c]Sensitivity to mefenoxam

previously, 1 h before inoculation of the pathogens (Debieu et al. 1995; Délye et al. 1997c). Control experiments were carried out by applying an equal quantity of sterile water onto leaf discs. No phytotoxicity of the different suspensions was observed with the doses used in the experiments. Four independent experiments were done with various isolates of powdery mildew and three with various isolates of downy mildew. Inoculations were carried out as described above with a *P. viticola* spore suspension at 5000 sporangia ml^{-1} (three drops of 15 µl per leaf disc), or under sterile conditions by blowing spores of *E. necator* from sporulating leaves (1000 conidia per cm^2 of leaf). Inoculated leaf discs were incubated for 7 and 12 days respectively as described above. Disease intensity was estimated by measuring growth and intensity of fungal mycelium and sporulation, as described previously (Corio-Costet et al. 2011; Debieu et al. 1995), and was expressed as the mean ± standard deviation. Dose–response curves for pathogens were used to determine EC_{50} (effective concentration inhibiting growth at 50 %) as described previously (Sombardier et al. 2011).

Evaluation of direct toxicity on *P. viticola* and *E. necator*

Direct toxicity on P. viticola

Sporangia suspensions were prepared at 150 000 sporangia ml^{-1} in product suspensions: ASM from 0.47 to

4.72 mM (100, 250, 500 and 1000 mgl^{-1}), FOS at 1.13 mM (400 mgl^{-1}), PK_2 at 0.63 and 6.37 mM (100 and 1000 mgl^{-1}). A negative control was carried out with water and a positive control with a fungicide (copper hydroxide, Champ Flo® at 100 mgl^{-1}). Sporangia suspensions were incubated 1 h at 4 °C and diluted at 1/10 before inoculation by drops of 15 µl of water, leading to a suspension at 15 000 sporangia ml^{-1} in 0.047 to 0.472 mM of ASM, 0.113 mM of FOS and 0.063 to 0.637 mM of PK_2.

Assessment of toxicity was made as described above, by visual evaluation of the percentage of growth and by assessment of spore production after 8-days incubation. The quantification of spore production was conducted using a non-destructive method for analysis of images where digital photos of sporulated leaf discs after 8-days growth were taken in natural light conditions. The images registered in JPEG format were treated with the image analysis software ImageJ version 1.43q (http://rsb.info.nih.gov/ij/) as described by Peressotti et al. (2011).

Direct toxicity on E. necator

One ml $dish^{-1}$ of each ASM, FOS and PK_2 suspensions was sprayed with a micro-diffuser under sterile conditions onto the upper surface of leaf discs 4 days after *E. necator* inoculations. Discs were incubated at 22 °C in a growth chamber as described above. Controls with sterile water or with a reference fungicide (Myclobutanil, Systhane®, at 100 mgl^{-1}) were carried out. Direct toxicity was assessed by visual evaluation of the percentage of growth as described above, 8 days after treatment (namely 12 days after inoculation).

Spore production was assessed for powdery mildew with a particle counter (Coulter Counter® Multisizer™ 3; Beckman Coulter, France). Sporulating discs were placed in vials filled with 15 ml isoton (Beckman Coulter® Isoton II diluant), one drop of non-ionic dispersant (Nacconol 90F, Dispersant Type IIIA; Beckman Coulter) and shaken. Spore production was thus assessed by counting the number of particles with diameters between 18 and 37 µm in a sample of 500 µl.

Statistical analyses

Three or four independent experiments of eight replicates were performed with the different isolates. Dose–response curves for pathogens were used to determine EC_{50} (effective concentration inhibiting growth at 50 %) as described in Sombardier et al. 2011. All data were subjected to an analysis of covariance by general linear model using the statistical programme Systat 11 (Systat Software, Inc) and significant differences were determined by Tukey's honest significant difference (HSD) test at level of $P \leq 0.05$.

Results

Effect of ASM concentrations on *E. necator* and *P. viticola*

Before assessing the direct effect on sporulation and mycelial growth of pathogens, ASM concentration ranges were tested to determine effective doses against the two pathogens. The ASM was effective against growth of 18 monospore isolates of powdery and six downy mildews isolates (Table 1) exhibiting different phenotypes and genotypes and led to 90 to 100 % growth inhibition (Fig. 1). Growth of *P. viticola* and *E. necator* isolates were progressively inhibited by increasing the ASM concentration from 0.47 to 7.08 mM (100 to 1500 mgl^{-1}). The EC_{50} values of ASM for the two groups of downy mildew, resistant (*Pv*-R) or sensitive (*Pv*-S) to fungicides, were not significantly different (Table 2). Similarly, the EC_{50} for the two genetic groups A (*En*-A) and B (*En*-B) of *E. necator* were not different. ASM treatment was significantly more efficient against *P. viticola* than *E. necator* at low concentration (~ 0.5 mM) (P=0.041, Fig. 1). Despite the EC50 difference, no significant difference concerning ASM efficacy on the various pathogens was observed (P=0.917). This study does not allow a conclusion on the ASM mode of action (direct or indirect). To distinguish the direct effect on the pathogen of an indirect effect on the stimulation of grapevine defences, an efficient dose of ASM (> EC_{60}), determined in the previous experiment, was applied either on mycelial growth of *E. necator*, or by incubation of *P. viticola* sporangia in ASM solutions before inoculation. No direct significant effect on *E. necator* growth and spore production (P=0.834, Figs. 2 and 3) at 1.90 mM of ASM was observed when the product was applied curatively after 4 days of growth. Concerning *P. viticola*, ASM also had no significant effect on spore production and mycelial growth

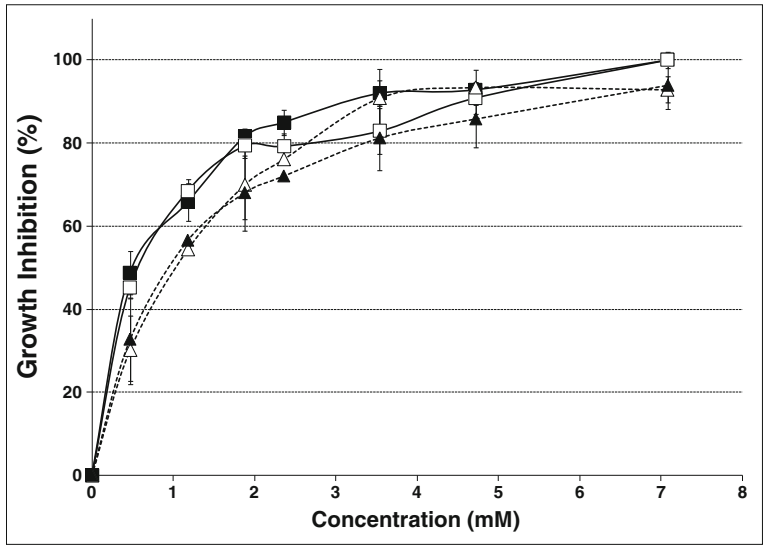

Fig. 1 Growth inhibition of *E. necator* and *P. viticola* after leaf treatment with different concentrations of ASM. Data corresponded to the mean of at least three independent experiments for *E. necator* (*dotted lines*, *triangle*) with N=10 for group A isolates (*white*) and N=8 for group B (*black*), and *P. viticola* (*full lines*, *square*) with N=2 for fungicide-sensitive isolates (*white*) and N=4 for fungicide- resistant isolates (*black*)

(P=0.420) (Figs. 2 and 4) at 1.90 mM, a concentration corresponding to ~ EC_{80} when the treatment with ASM was applied before inoculation (Fig. 1).

Effect of FOS concentrations on *E. necator* and *P. viticola*

As above, a FOS concentration range was used to determine effective doses on the two pathogens. Throughout this study six isolates of *P. viticola* and 18 isolates of *E. necator* were tested independently at least three times to evaluate the EC_{50}. Overall, as expected, FOS was more effective against *P. viticola* than against *E. necator* (Fig. 5, Table 2). The EC_{50} values for the fungicide- sensitive isolates and for the fungicide- resistant isolates of *P. viticola*, were not significantly different (P=0.261, Table 2). Most surprising was the sensitivity to FOS of the two genetic groups of *E. necator* that was significantly different (P=0.000). It is apparent that the EC_{50} value was higher (3.30 mM) for the isolates belonging to genetic group A compared to the EC_{50} value of the isolates belonging to genetic group B (0.89 mM). FOS was more effective (3.70 fold more) against genetic group B of *E. necator* than against group A isolates. The EC_{50} of group B isolates of *E. necator* was not significantly different from the EC_{50} of the two groups of *P. viticola*.

In the second experiment to demonstrate a potential direct effect, conversely to the ASM effect, strong evidence of the direct effect of FOS on *E. necator* was found with a significant decrease in spore production of 38.7 % (P=0.007), compared to the control and to the reference fungicide myclobutanil which led to 97.3 % inhibition (Fig. 3). This inhibition is similar to that observed in Fig. 5, on genetic group B, suggesting a direct effect of FOS on *E. necator* growth. The incubation of sporangia of *P. viticola* in FOS at a concentration of 1.13 mM (500 mgl^{-1}) induced an inhibition of 87.5 % of downy mildew (Fig. 4), similar to the fungicide reference inhibition and similar to the inhibition obtained in Fig. 5 with FOS applied on leaves. FOS had a direct effect on the production of sporangia and/or mycelial growth of grapevine downy mildew.

Table 2 Effective concentrations inhibiting growth at 50 % (EC_{50}) obtained from dose–response curves. Letters represent the significantly different values at the threshold of 0.05 %

Mildew isolates	EC_{50} (mM ± SEM)		
	ASM	FOS	PK_2
P. viticola- Fungicide- resistant	0.47^{a}±0.06	0.52^{a}±0.04	1.27^{a}±0.08
P. viticola- Fungicide- sensitive	0.53^{a}±0.13	0.48^{a}±0.07	0.64^{a}±0.02
E. necator- Group A	1.05^{a}±0.28	3.30^{b}±0.46	7.44^{b}±1.38
E. necator - Group B	0.95^{a}±0.10	0.89^{a}±0.32	6.36^{b}±1.53

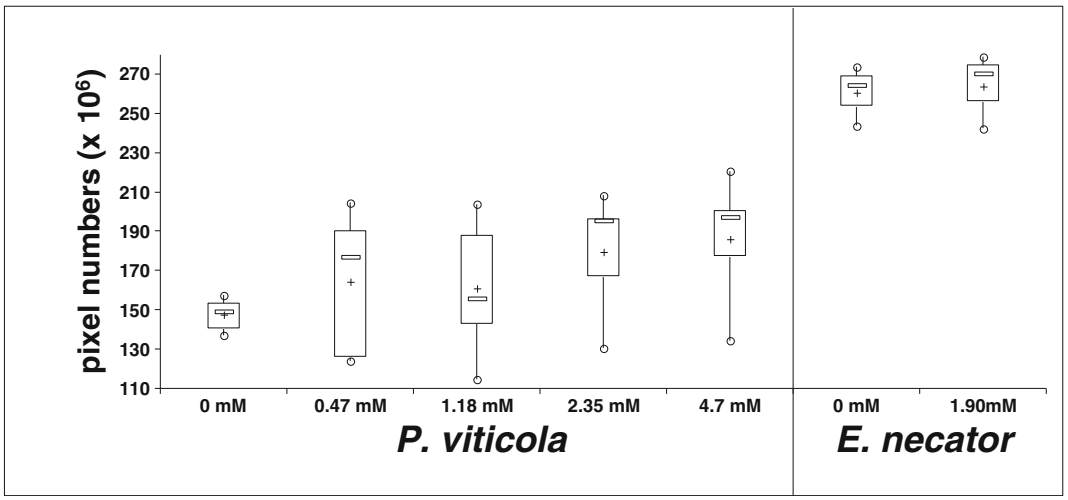

Fig. 2 Direct effect of ASM on *P. viticola* and *E. necator* growth and spore productions. Box plot representations of pixel numbers obtained after image analysis

Effect of fertilizer PK_2 concentrations on *E. necator* and *P. viticola*

It is apparent from Fig. 6 that the application of concentrations of PK_2 higher than 4 mM to the different isolates led to a growth inhibition of 100 % for downy mildew isolates but only to 65 % for powdery mildew isolates. As found with FOS, the EC_{50} values obtained for the fungicide-sensitive and resistant isolates of downy mildew (Table 2), were also not significantly different (P=0.224). However, at lower concentrations of PK_2 (e.g., 0.63 mM) applied to the two groups, the effectiveness tended to be better against fungicide-sensitive isolates than against fungicide-resistant isolates of *P. viticola* (Fig. 6). Conversely to the FOS results and with regard to the EC_{50} of PK_2 obtained on groups B and A of *E. necator*, no significant difference was observed (P=0.247) (Table 2). Nevertheless, PK_2 was significantly more effective (P=0.000) against *P. viticola* than against *E. necator* isolates. But as above, it was not possible to conclude on the PK_2 mode of action (direct or indirect).

Through the application of PK_2 on *E. necator* mycelium growth (curative effect), or preparing sporangia suspensions of *P. viticola* into a PK_2 solution (direct inhibition), we could discern the direct effect of the product from the indirect effectiveness due to plant defence stimulation. In this experiment, PK_2 had a significant effect on *E. necator*, leading to complete inhibition of spore production (100 %), whereas the reference fungicide, myclobutanil, led to 97.3 % (Fig. 3), suggesting a direct toxicity of the product on mycelial growth and spore production. This point was reinforced by the fact, that in the first experiment with a PK_2 application on leaves before inoculation (Fig. 6) at a similar dose, we got only about 30 % inhibition. Concerning *P. viticola*, two concentrations of PK_2 were tested, and the incubation of sporangia in solution at 0.63 mM of PK_2 (100 mgl^{-1}) had no effect on development of *P. viticola*, but at 6.37 mM

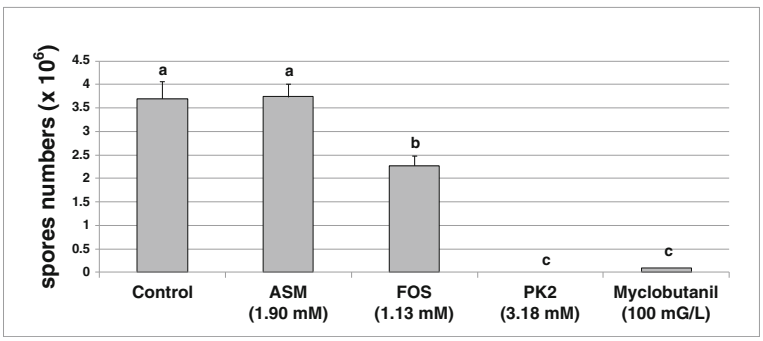

Fig. 3 Spore quantification of *E. necator* with a particle counter. Statistical differences are shown by different letters at the threshold of 0.05 %

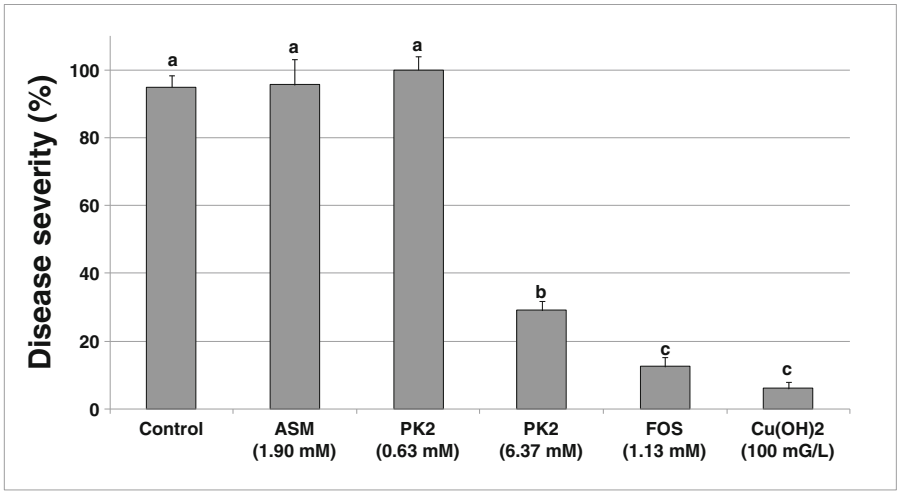

Fig. 4 Disease intensity by measuring growth and intensity of of *P.viticola* sporulations. Sporangia suspensions were incubated with different compounds 1 h before dilution at 1/10 and inoculation. Copper hydroxide (Champ Flo®) at 100 mgl^{-1} and distilled water were used as positive and negative controls, respectively. Statistical differences are shown by different letters at the threshold of 0.05 %

(1000 mgl^{-1}), PK_2 induced a 70.9 % inhibition of downy mildew growth. These results showed that PK_2 had a direct effect on the two pathogens.

Discussion

The purpose of the current study was to determine the sensitivity of various isolates of grapevine mildews (*P. viticola* and *E. necator*) exhibiting different genotypic and/or phenotypic characteristics to compounds potentially stimulating plant defences such as ASM or phosphonates. It was interesting to look at the behaviour of isolates more or less adapted to the presence of pesticides (sensitive plant *vs* resistant) with these molecules potentially involved in grapevine defence and also to be able to show a more or less significant direct efficacy on different pathogens. Amongst the phosphonates, one was used as a fertilizer (PK_2) and the second as a fungicide against oomycetes (FOS). They are also described as inducers of systematic acquired resistance to oomycetes (Daniel and Guest 2006; Eshraghi et al. 2011; Guest 1984; Massoud et al. 2012) as is ASM which is also a plant defence activator (Friedrich et al. 1996; Lawton et al. 1996; Dufour et al. 2012). One of the more significant findings to emerge from this study was that, depending on the mildew species, or on the genetic group intra-species under consideration, the efficacy of the treatments on grapevine leaves led to very different results. The use

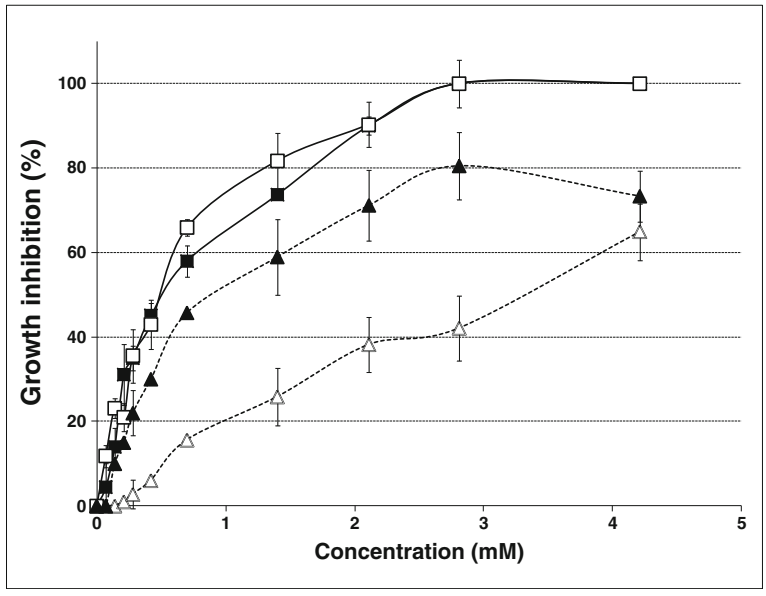

Fig. 5 Growth inhibition of *E. necator* and *P. viticola* after leaf treatment with different concentrations of FOS. Data corresponded to the mean of at least three independent experiments for *E. necator* (*dotted lines*, *triangle*) with $N=10$ for group A isolates (*white*) and $N=8$ for group B isolates (*black*), and *P. viticola* (*full lines*, *square*) with $N=2$ for fungicide- sensitive isolates (*white*) and $N=4$ for fungicide- resistant isolates (*black*)

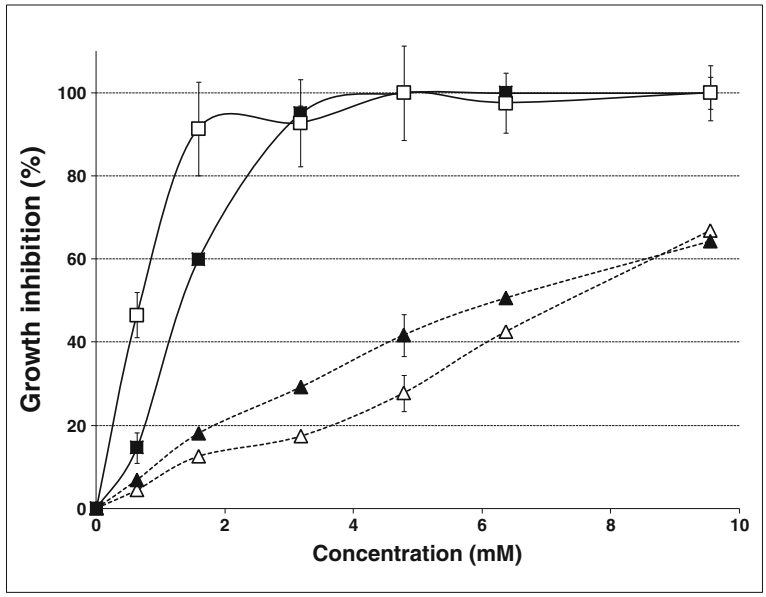

Fig. 6 Growth inhibition of *E. necator* and *P. viticola* after leaf treatment with different concentrations of PK_2. Data corresponded to the mean and SE of at least three independent experiments for *E. necator* (*dotted lines*, *triangle*) with $N=10$ for group A isolates (*white*) and $N=8$ for group B isolates (*black*), and *P. viticola* (*full lines*, *square*) with $N=2$ for fungicide- sensitive isolates (*white*) and $N=4$ for fungicide- resistant isolates (*black*)

of these products showed that, even belonging to the same chemical family (e.g., phosphonates), different efficacies were obtained. The relevance of this was clearly supported by the difference in sensitivity of the two genetic groups of *E. necator* to FOS and to PK_2. Indeed, although PK_2 showed similar efficacy on the two populations of *E. necator*, FOS, at concentrations included between 0.5 to 3 mM, exhibited greater efficacy on the isolates belonging to group B, not significantly different from that observed for the downy mildew isolates. This difference of sensitivity of genetic group A to FOS suggests that group A isolates, although usually more sensitive to fungicides, would be less sensitive to the action of a phosphonate combined with the ion aluminium. As no difference was observed with the second phosphonate tested (PK_2), we suggest that group A isolates could be less sensitive to the action of the ion aluminium, perhaps in relation to the aggressiveness of the group A isolates. Indeed, group A is less aggressive in terms of germination and infection efficiency, but more aggressive than group B in terms of latency period, lesion diameter and spore production (Montarry et al. 2008).

The present study suggested that spraying phosphonates, or ASM, on grapevine leaves before inoculation, was effective in reducing powdery and downy mildew development, and that pathogen growth progressively decreased in relation to the increase in compound concentrations. Nevertheless, overall, downy mildew isolates were more sensitive to phosphonates and ASM than powdery mildew isolates (Table 2). ASM protected grapevine leaves effectively whatever the pathogen, whereas the phosphonates exhibited a significantly better efficacy against *P. viticola* populations. On the other hand, an experiment performed in the vineyard showed that the ASM led to better protection against downy mildew on clusters than on leaves and conversely, that the FOS led to better protection on leaves than on clusters. As expected, protection was better against *P. viticola* than against *E. necator* with phosphonate in vineyard (Dufour 2011).

As mentioned in the literature (Siegrist et al. 1997), no direct effect of ASM on *P. viticola* and *E. necator* was observed on mycelial growth and spore production, contrary to the ASM effect observed on *B. cinerea* at high concentrations (1 to 3 gl^{-1}) (Munoz et al. 2010). By contrast, fungitoxicity was obtained after use of phosphonates on *P. viticola*, but also on *E. necator*. The efficacy against *E. necator* did not support the paper by Speiser et al. (2000) who wrote that potassium phosphonate was effective against downy mildew, but not against powdery mildew. On the other hand phosphonates are known to inhibit spore formation and germination in oomycetes species (Guest and Grant 1991; Coffey and Joseph (1985), Farih et al. 1981). In our experiment, the mean EC_{50} of FOS was 177 mgl^{-1} and the direct action on *P. viticola* at 400 mgl^{-1} (1.13 mM) 1 h before dilution (40 mgl^{-1}, 0.11 mM) and release on the encystement of

zoospores, could be consistent with the effects of FOS on various *Phytophthora* species (*capsici*, *citrophthora and parasitica*) as described by Matheron et al. (2000). The authors showed that depending on the FOS concentration, the phosphonate inhibited spore formation (3 to 12 mgl^{-1}), mycelial growth (30 to 103 mgl^{-1}) or motility and germination of zoospores (300 to 1000 mgl^{-1}). It seemed that FOS and PK_2 reduced sporangia mobility and/or zoospore release. After 1 h of incubation in phosphonate solutions at 1.13 mM and 6.37 mM, respectively, the suspensions of sporangia were diluted 10 times before the drop deposits of *P. viticola* sporangia which were in the presence of 0.13 and 0.63 mM of phosphonates up to the encystement of zoospores. Finally, 8 days later, we observed a sporulation inhibition of 87.5 and 72 % for FOS and PK_2, respectively, with PK_2 being less effective than FOS on *P. viticola*. The present study also provides additional evidence with respect to the direct effect of phosphonates on powdery mildew spore production, with FOS and PK_2 exhibiting a significant effect on spore production (38.7 and 100 % inhibition). The current investigations on growth and sporulation were limited by the obligate nature of the pathogens and we were only able to examine indirectly the effect of products on plant infection by *E. necator*. Powdery mildew and downy mildew are obligate pathogens that can only multiply on their plant host. The efficiency of the tested products corresponds in fact to a cumulative effect between a possible direct toxic effect on the fungi and/or the defence elicitation of the grapevine, which could not easily be separated from one an other in the case of these strictly obligate parasites.

Concerning the effect of ASM, our study once again reinforced the fact that ASM acts on the elicitation of plant defences. The mechanisms of ASM-induced plant resistance have been shown to involve the activation of SAR mechanisms based on the salicylic acid (SA) pathway (Friedrich et al. 1996; Lawton et al.. 1996) with subsequent up-regulation of defence genes and an accumulation of phenolic compounds (Iriti et al. 2004). Recently, we deciphered the effect of ASM on grapevine defences and we found that, after ASM treatment, grapevine protection was clearly related to the induction of PR-protein genes (e.g., *PR-1*, *PR-2*, *PR-3*, *PR-10*, *PR-8* and *PR-6*), to the over-expression of genes repressed by *P. viticola* and *E. necator* in infected leaves, and to the production of pterostilbene (Dufour et al. 2012) according to studies carried out on different plant-pathogen systems (Brisset et al. 2000; Perazzolli et al. 2008; Hukkanen et al. 2008; Bovie et al. 2004). The protective effect of ASM on the development of *Rhizoctonia solani* disease in rice was noticeable if it was applied to leaves 24 h before inoculation (Rohilla et al. 2001). Our study provided evidence that ASM and phosphonates applied 1 h before pathogen inoculation induced efficient grapevine protection against downy and powdery mildew, and that this time lapse was sufficient. Further work needs to be done to establish whether plant stimulators should be applied in preventive application and to look at their curative potential. This study means we have a more precise idea of doses to apply in the vineyard. In controlled conditions, the ASM dose giving 90 % efficacy (EC_{90}) against downy and powdery mildew was approximatively 6 mM. Similarly, the EC_{90} of FOS and PK_2 against downy mildew was 2.5 mM, whereas against powdery mildew, this dose was higher (more than 4.21 and 9.55 mM, respectively) explaining why the phosphonates are described as anti-oomycetes.

In conclusion, with this study we provide results of the direct effects on inhibition of growth of *E. necator* and *P. vitiocola* by two phosphonates (FOS used as a fungicide and PK_2 used as a foliar fertilizer) as well as the possible indirect effects of the products on the stimulation of grapevine defences. For the first time, we have shown that depending on the variability of the pathogen (inter- or intra-species), the efficacy of these compounds can be very variable. The difference observed, according to whether they belong to group A or B of powdery mildew, is an original result that could be explained by a lesser sensitivity of group A isolates to the action of the ion aluminium, and/or by the aggressiveness of these isolates which produce more spores and lesions than B isolates. We corroborate the fact that ASM exhibits no direct effect on the two pathogens and the efficacy observed may result from an induction of grapevine defences. The findings of this study could have important implications for developing the use of plant defence activators which may or not have a double mode of action.

The use of products such as phosphonates or ASM with direct or indirect action and able to stimulate grapevine defence could help to limit the appearance of resistance to uni-site fungicides and reduce the use of fungicides harmful to the environment. To develop and support sustainable viticulture, the use of plant

defence activators, may represent a promising low-impact tool for the control of grapevine diseases. However, our study shows that the diversity of pathogens can have an impact on the efficacy of these compounds. More research on the diversity of pathogen populations and the effect of these compounds on different varieties of grapevine needs to undertaken before developing new pest management strategies. Although effective stimulation of plant defences shows good, reproducible, results under laboratory conditions, the use of these compounds in the vineyard is more difficult and usually shows disappointing results. Further research should be done to investigate the use of phosphonates and ASM in the vineyard. This is an important issue for future research and for optimization of their use in commercial vineyards.

This study also gives us a more precise idea of doses to be applied. Although in controlled conditions, the minimal dose providing 90 % efficiency of ASM (EC_{90}) against downy and powdery mildew was approximately 6 mM. That of FOS and PK_2 against downy mildew was 2.5 mM, whereas against powdery mildew, the EC_{90} was never reached (more than 4.21 and 9.55 mM respectively). During a trial implementation to assess efficiency of these products in the vineyard, it would be sensible to apply doses higher than or equal to these EC_{90}.

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