Comparison of phosphonate derivatives (fosétyl-Al, PK2) efficacy to that of BTH as grapevine defence elicitors against *Plasmopara viticola*

M. C. Dufour[a] and M. F. Corio-Costet[a]

[a] INRA, Bordeaux, UMR Santé Végétale (1065) , BP, 81, 33883 Villenave d'Ornon, France.

Since the introduction of the causal agent of grapevine downy mildew (*Plasmopara viticola*) from America in 1878, the control of outbreaks requires numerous chemical treatments having consequences on the environment and the health. One of the means of reducing the use of chemicals is to modify agricultural practices. It is thus urgent to develop and to support the sustainable agriculture that it is integrated and\or agro-biological. So stimulating defences, in addition to resistant varieties and biological warfare, is an interesting method of alternative pest management.
Because they act on the plant and not directly on the pathogenic, elicitors lead to multifactorial resistance of the plant host which means that pathogen should find it more difficult to get round.

The UMR Santé Végétale unit at INRA Bordeaux ha developed studies to work out new strategies integrating the use of additional methods (biological, plant selection, or elicitors) in pest management control. The use of plant defence stimulators, apart from seeing reasoned use of plant treatment products, seems promising.

It is well known that phosphonates have a powerful antifungal activity and the fungicide fosetyl-Al (*O*-ethyl phosphonate) (1, 2) is known to exert both a direct effect on the pathogen and an indirect effect via stimulation of host defences. In this present study, the efficiency of two phosphonate derivatives, Fosetyl-Al and a foliar fertilizer (PK2) (3) was compared to Benzothiadiazol (BTH) (4), a salicylic acid analogue, against *Plasmopara viticola* (5).

The assessement of efficacy was made at several levels, biological (efficacy on pathogen growth), biochemical (quantification of phenolic compounds) and molecular (gene expressions). The aim was to understand better how these elicitors work, and also to provide some answers with regard to the interest of developing alternative strategies for induction of plant defence during the growing season in vineyard. Trials in the vineyard, on experimental plots treated with the various compounds, were carried out to estimate the efficacy of elicitation methods under natural conditions.

Material and Methods

Leaf disks of Cabernet Sauvignon pretreated with a range of concentration of PK2, Fosetyl and BTH, and inoculated with 4 different strains of downy mildew, were used to check the potential ED_{50} of elicitors. Grapevine defence efficacy assessment were carried out on leaf disks and controls were carried out by sprinkling sterile distilled water. Each elicitor was applied at 6 dilutions on eight disks by pulverization with the various concentrations from 0 to 7 mM. Three independent experiments were performed with downy mildew strains.
Treatment efficacy was determined by visual assessment of percentage of growth of the pathogen on the leave disks after 7 days at 22°C. The results were presented as an average percentage of inhibition compared of fungus growth compared to control, using the following calculation:

$$\% \text{ growth inhibition} = 100 \times \left(\frac{\%\text{"treated" growth}}{\%\text{ "control" growth}} - 1 \right)$$

By means of graphic representations of the percentage of inhibition according to compound concentrations, a value of Effective Dose 50 (ED50) corresponding to the dose inhibiting in 50 % the growth was determined.

Secondly, leaves were pre-treated with the various compounds 24 hours before pathogen inoculation and gene expression levels of 20 genes known to play a part in plant defences mechanisms were quantified by RT-PCR for three days after the pathogen inoculation. We followed the expression of genes coding for enzymes of phenylpropanoïd biosynthesis (PAL, STS, CHS, CHI, LDOX and BAN), phytohormone biosynthesis (JA, SA and ethylene) LOX, ACC PAL and genes coding for PR proteins (CHIT4c, PGIP, PIN, GLU, PR1 and PR10).
RNA was isolated from frozen tissues samples by phenol-chloroform extraction followed by lithium chloride precipitation according to the method of Reid and al. (2008). The extraction buffer contained 300 mM Tris HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVPP, 0.05% spermidine trihydrochloride, and just prior to use, 2% β-mercaptoethanol. Tissue was ground to a fine powder in liquid nitrogen and was added to pre-warmed (65°C) extraction buffer at 20 ml/g of tissue. Tubes were subsequently incubated in a 65°C water bath for 10 min and shaken. Mixtures were extracted twice with equal volumes chloroform:isoamyl alcohol (24:1) then centrifuged at 3,500 × g for 15 min at 4°C. To the supernatant, 0.1 vol 3 M NaOAc (pH 5.2) and 0.6 vol isopropanol were added, mixed, and then stored at -80°C for 30 min. Nucleic acid pellets were collected by centrifugation at 3,500 × g for 30 min at 4°C and dissolved in 1 ml Tris-EDTA Buffer (pH 7.5). After a selective precipitation of RNA, 0.3 vol of 8 M LiCl was added and the sample was stored overnight at 4°C. RNA was pelleted by centrifugation at 20,000 × g for 30 min at 4°C then washed with ice cold 70% EtOH, air dried, and dissolved in DEPC-treated water. RNAs were quantified after DNase I digestion by absorbance measure at 260 nm and 280 nm and 260/280 nm ratios were determined (Genequant pro, Amersham Bioscience, France). cDNAs were synthesized by reverse-transcription using 2 µM oligo $d(T)_{15}$ and using Promega reagents. The reverse transcription reaction mix is assembled on ice to contain nuclease-free water, M-MLV reverse transcriptase (Promega) and its 5X reaction buffer, dNTPs, and ribonuclease inhibitor according to the manufacturer's instructions. An initial annealing at 94°C for 5 min, the

reaction is incubated at 42°C for 1hr. The obtained cDNA was stocked at -20°C.

Several common housekeeping genes were selected for expression analysis (Table 1).

Real-time PCR reactions were determined using 96-well plates with an iCycler thermocycler (Bio-Rad france, Ivry sur Seine) using SYBR® Green. For each reaction, 1µl of each primer at 10nM, and 7 µl of 2X- Blue SYBR Green fluorescein Mix including Hot start DNA polymerase, dNTP and $MgCl_2$ (Abgene, France) and 5µl of cDNAs, were used in compliance with manufacturer's instruction. Amplifications were measured under the following conditions: a first step at 94°C for 15 min, and 40 cycles at 94°C for 10 s, 55°C for 10 s, and 72°C for 20 s. Relative gene expression was obtained with the formula: fold induction = $2^{-[\Delta\Delta Ct]}$, where ΔΔCt = [Ct GI (unknown sample) - Ct EF1γ (unknown sample)] - [Ct GI (reference sample) - Ct EF1γ (reference sample)]. GI is the gene of interest, and EF1γ is the grapevine Elongation Factor1 γ gene used as the internal control. The reference sample is the sample chosen to represent 1 fold expression of the gene of interest (e.g., control leaves untreated and no inoculated). Each sample was assayed in duplicate at the minimum.

Table 1. Primer sets used for the grapevine defence genes expression.

Gènes	N° accession GeneBank
Chaine gamma du facteur d'élongation 1 (**EF1γ**)	AF176496
Phénylalanine ammonia lyase (**PAL**)	X75967
Stilbène synthase (**STS**)	X76892
PR protéines de classe 1 (**PR1**)	AJ536326
PR protéines de classe 10 (**PR10**)	AJ291705
Chalcone isomérase (**CHI**)	X75963
Chalcone synthase (**CHS**)	X75969
Chitinase de classe III (**CHIT3**)	Z68123
Chitinase de classe IV (**CHIT4**)	VVU97521
Protéine inhibant les polygalacturonases (**PGIP**)	AF305093
β 1,3 glucanase (**GLU**)	AF239617
Inhibiteur de sérine protéase (**PIN**)	AY156047
Lipoxygénase (**LOX**)	AY159556
Leucoanthocyanidine dioxygénase (**LDOX**)	X75966
Glutathione S- transférase (**GST**)	AY156048
Anthocyanidine réductase (**BAN**)	VVI000166
Acide 1-aminocyclopropane, 1-carboxylique synthase (**ACC**)	AF424611
Antranilate Synthase (**ANTS**)	XM 002281597
Chorismate Mutase (**CHORM**)	FJ604854
Chorismate Synthase (**CHORS**)	FJ604855

Experimental field tests were carried out on an experimental plot of land, established by the repetition of 4 blocks consisting of three vine stocks of Cabernet Sauvignon, treated weekly with 2 g /L of BTH, 2.5 g/L de fosetyl and 2,1 g/ L of Dithane (reference fungicide). Treatments began on May 5th, 2009 at the stage 13-14 of the BBCH scale (3-4 leaves unfolded) and stopped on July 21st at the stage 79 of the BBCH scale (berries reach their final size), namely 12 treatments. Untreated control blocks were also introduced into the experimental plot. An artificial inoculation of *P. viticola* was carried out on May 27th, (stage 55, BBCH), 24 hours after the fourth treatment, at the rate of 6 leaves by vine stock pulverized with a solution of 45 000 sporangia per ml.

The development of the disease was then measured every week by estimating the percentage of leaves attacked and the severity on leaves.

Results:

Laboratory assays showed that BTH, Fosetyl and PK2 were uniformly effective against *P. Viticola* (Figure 1).

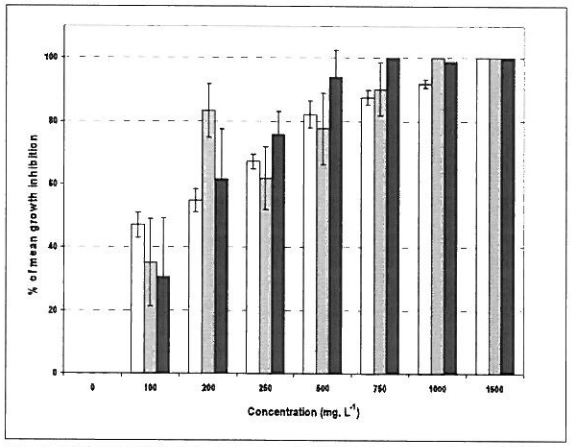

Fig.1. Effect of BTH (white bars), FOS (grey bars) and PK2 black bars) on *Plasmopara viticola* growth.

Leaf disks were inoculated 1 hour after being sprayed with different concentrations of BTH (white bars), FOS (grey bars) and PK2 (black bars). Data were the mean of 2 independent experiments.

We obtained no significant differences in efficacy (P = 0.917) between the various compounds.

BTH, Fosetyl-Al and PK2 all showed good efficacy against downy mildew and allowed to 100 % of inhibition (figure 1). The values of ED_{50} were 0.47 mM, 0.50 mM and 0.96 mM, respectively. However no significant difference existed between the various groups (data not shown).

Concerning the level of gene expression, no significant difference between the control and the treated with the various products in the absence of pathogens was detected. A significant repression of the expression of the majority of the genes was observed after inoculation with strains of pathogens alone ($P < 0.05$, data not shown). Significant differences were also found in leaves pretreated with the various elicitors and in the presence of the pathogen. In the presence of BTH with downy mildew, PR proteins were specifically overexpressed (PR1, GLU, LOX and CHIT3) (Table 2).

Table 2. Balance of significant overexpressed genes depending on different treatments.

	PK 2			FOS			BTH		
	24 hpi	*48 hpi*	*72 hpi*	*24 hpi*	*48 hpi*	*72 hpi*	*24 hpi*	*48 hpi*	*72 hpi*
CHI	**27,0**	4,2	2,0	1,7	9,8	1,6	0,4	6,3	1,6
CHS	1,2	1,6	3,2	1,1	2,8	**13,4**	0,5	2,2	2,3
LDOX	0,1	5,8	1,5	0,1	3,5	0,4	**0,0**	2,1	3,5
PR1	7,4	10,9	2,5	1,8	6,4	**22,2**	4,6	**93,1**	2,6
PR10	**14,0**	4,4	1,8	**6,5**	8,9	2,6	6,2	9,2	2,0
CHIT3	0,7	1,8	0,6	2,0	3,2	2,3	4,6	**9,7**	0,2
CHIT4	**33,3**	4,1	5,5	**14,4**	4,7	3,5	2,6	8,9	5,4
PGIP	**26,1**	0,6	**3,3**	16,2	10,4	3,9	0,2	3,6	0,9
GLU	**0,1**	1,0	1,1	7,7	**3,2**	2,3	0,7	**5,7**	0,1
PIN	4,4	**8,0**	**7,4**	0,5	7,1	4,5	2,5	13,9	3,4
LOX	1,5	0,7	3,1	2,1	1,9	0,8	0,6	**2,9**	0,0

Concerning phosphonates, they essentially led the over expression of genes coding for the anthocyanin biosynthesis

(CHI, CHS) and for PR proteins (PR1, PR10, PGIP, GLU and CHIT4) in the presence of *P. viticola* (Table 2).

The field trial carried out in 2009 revealed that the outbreak began its development from June 18th with a new phase taking place from July 9th until August 20th when more than 90 % of leaves control plots were attacked (figure 2A and 2B). On grapes, more than 90 % severity was reached in July. No grapes remained at the date of September 1st in control plots.

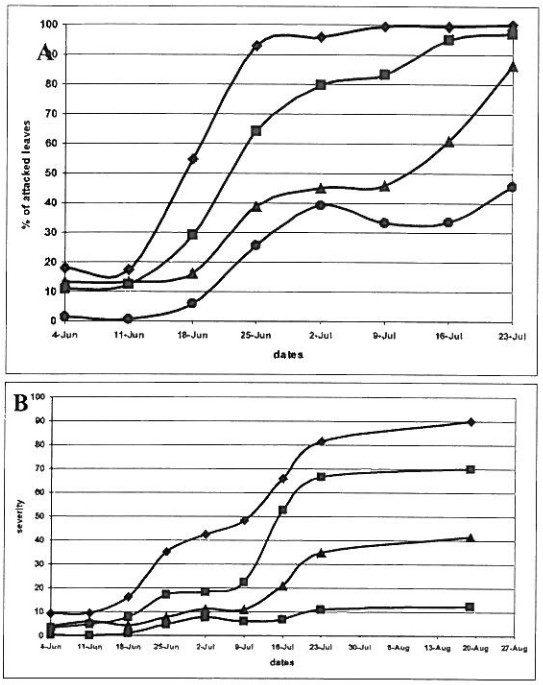

Fig. 2. Evolution of downy mildew outbreak in 2009 for the 4 product tested (BTH (square), FOS (triangle), Dithane (circle) and Control (diamond)). **(A)** % of attacked leaves, **(B)** severity (% of mean attack).

Under experimental conditions, the reference fungicide, showed good efficacy throughout the experiment, on both leaves and grapes.

Fosetyl also presented a good efficacy until July 16th and showed an attack on leaves of only 40 % at August 20th in comparison with the control where it was more than 90 %. For grapes, 20 % of grapes were affected in June, and in July the mean percentage of grape attack reached 70 %. By September 1st, some grapes remained but were very affected. BTH also provided good protection until July 9th, but leaves were then very quickly affected and severity reached 70 %. On the contrary, for grapes in June severity was only 20 %, as with Fosetyl, but on the other hand, in July better protection was conferred by BTH than with Fosetyl. Indeed BTH lead to 40 % of severity on grapes against 70 % with Fosetyl. It should be noted, however, that the vines treated with BTH still had fine, undamaged grapes in the date of September 1st, but with delayed ripening.

Conclusion:

This study showed that the answers of the plant after various elicitor treatments lead to efficacies and to modulation of gene expression depending on the product used. So, BTH, a salicylic analogue, favours the over expression of PR proteins such as PR1, chitinase 3 and Glucanase. On the contrary, phosphonates lead to over expression of the anthocyanin biosynthesis pathway, and also of PR proteins such as PR10, Chitinase 4, Polygalacturonase in the presence of *P. viticola.* All in all the downy mildew leads to gene overexpression involved in anthocyanin biosynthesis s and PR proteins.

The methods developed in our study to assess the stimulating effect and the efficacy of elicitors enabled us to obtain a better understanding about the real potential of elicitors against downy mildew. To complete the assays, biochemical analysis of phenylpropanoids is in progress. The possibility of following the outbreaks and gene expression in vineyard conditions also enlightened us as to the real state of grapevine defence after elicitation and before or after attacks of the pathogen. The field experiment carried out in 2009 will be carried out again in 2010. This will allow us to compare the real efficacy of products on downy mildew. The biochemical and molecular analyses of leaves taken at different times will be of interest to understand and assess the possibility of using elicitors as an alternative or additional method in pest management of the grapevine.

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