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"BioMolChem": a Tool to Assess the Defense Status of Grapevines after Stimulations or Not of Cultivar or Resistant Genotypes, from Genes to the Field

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Abstract

Stimulating plant defenses or resistant plant varieties is promising as an alternative method for limiting pesticide use in agriculture. To assess the defense status of the grapevine we have developed a triple approach called "BioMolChem".

The biological tests measure the efficacy of grapevine defenses against two major biotrophic pathogens (*Erysiphe necator*, *Plasmopara viticola*). Molecular assays by q-RT-qPCR show the expression patterns (over-expression or repression) of 20 genes involved in grapevine defenses, and they can then be correlated or not with the level of protection. Biochemical analyses of phenylpropanoides by HPLC are used to quantify and identify molecules of interest, and correlate them with specific gene expression (stilbene biosynthesis) and the acquired protection. This tool was tested on leaves ('Cabernet Sauvignon') after stimulation by different elicitors (benzothiadiazole, phosphonates), on grapevine, 'Cabernet Sauvignon', genotypes resistant to powdery and downy mildew, and in the vineyard. We obtained correlations between the expression of PR-protein genes and genes coding for biosynthesis pathways (stilbene, tryptophan) and with the level of protection. Similarly, we found correlations between the presence of known and unknown molecules and the level of protection. Resveratrol, a well-known phytoalexin of the grapevine, is a good marker of defense status but not of protection. Therefore, we now have available a tool for understanding the defense and protection status of the grapevine in laboratory and field experiments.

INTRODUCTION

Plants possess the ability to defend themselves against pathogens, but the success of this defense depends on the speed and intensity of their response, in addition to activation of the defense mechanisms. In the presence of a pathogen, grapevine triggers defense mechanisms with variable success depending on the degree of resistance of the cultivars, thus suggesting the basal induction of defenses (Fung et al., 2008). However, it is possible to induce some defenses in susceptible plants by using inducers that mimic natural signaling compounds and have no fungicide activity, such as acibenzolar-S-methyl, or benzothiadiazole (BTH), which are known to be effective against a broad spectrum of pathogens in various plants (Tally et al., 1999). Typically, this salicylic analog induces resistance associated with systemic acquired resistance (SAR) (Walters et al., 2007). Elicitors could be used to reinforce inherent defenses in susceptible or partial resistant cultivars. Vines cultivated in Europe are a perennial crop that is susceptible to many diseases including obligate parasites such as powdery (*Erysiphe necator*) and downy (*Plasmopara viticola*) mildew, which account for more than 70% of fungicides

used in viticulture. With a view to sustainable viticulture that limits chemical inputs, there is need to innovate and combine different methods such as biological control, plant breeding and the use of plant defense stimulators. To do this, it is necessary to understand the impact of the agro-system on the efficacy of plant stimulators and to have tools and markers for assessing the defense status of the plant and to explain its different responses observed in vineyard, where results with elicitors are often disappointing. Why is the efficacy of elicitors on plant defenses in the vineyard so variable? Various factors modulate the efficacy of plant defenses after treatment with a stimulator. Firstly, the pathogen itself can explain some of the part of variability, a factor that is often forgotten. The inter- or intra-species diversity, their aggressiveness and their ability to adapt to plant toxins can affect the efficacy of plant defenses. Secondly, the different levels of efficacy may be due to the plants themselves and their ability to defend themselves, which in turn depends on their genetic background. Another factor is ability of plants to mobilize their defense mechanisms. Furthermore, the organ considered and the age of plants also has an effect. Finally, environmental and climatic conditions and the bioavailability of the elicitor play a significant role on the overall system.

Stimulating grapevine defenses to make them less susceptible to pathogen attack is a real challenge, especially in the field. Within this framework, we have developed indicators called "BioMolChem" that provide information about the defense status of the grapevine.

MATERIALS AND METHODS

Grapevine plants (*V. vinifera* 'Cabernet Sauvignon') and various resistant genotypes arising from a cross between *Muscadinia rotundifolia* and 'Cabernet Sauvignon' obtained from INRA Colmar (D. Merdinoglu) were propagated in a greenhouse as described in Dufour and Corio-Costet (2013). *Plasmopara viticola* and *Erysiphe necator* isolates from the laboratory collection were multiplied on grapevine leaves and inoculated as described in Corio-Costet et al. (2011). Leaves were treated with benzothiadiazole (BTH) or phosphonates (fosetyl-Al and a fertilizer PK2) 24 hours before inoculation, as described in Dufour and Corio-Costet (2013). At each sampling time point (0, 24, 48 and 72 h) postinoculation, six treated or untreated leaves were sampled. The development of the disease was assessed seven days after downy mildew inoculation or 12 days after powdery mildew inoculation as described previously (Debieu et al., 1995; Corio-Costet et al., 2011). Assays performed with resistant genotypes were similar to those with the susceptible cultivar 'Cabernet Sauvignon' as described in Dufour (2011). For biological, biochemical and gene expression assays two independent experiments were carried out.

Expression experiments and RT-qPCR were performed with a series of 20 genes, including the gamma chain elongation factor 1 gene (EFγ1) as internal standard to normalize the template of cDNA. mRNA were extracted and q-PCR performed as described in Dufour et al. (2013). The extraction and quantification of stilbenes were performed as described in Belhadj et al. (2008). From each sample of 100 mg of dried leaves, analyses were carried out by HPLC as described in Dufour et al. (2013).

In natura, trials were carried out in a Bordeaux vineyard as described in Dufour (2011), on four blocks of 'Cabernet Sauvignon' for each trial. Treatments were applied between May and July with BTH, a phosphonate, or a reference fungicide. To obtain a significant pathogenic pressure, we artificially inoculated leaves with downy mildew in May. Epidemic monitoring was carried out on leaves and on clusters during the growing season.

RESULTS AND DISCUSSION

To investigate the defense status of grapevine better, we developed a triple approach combining biological, molecular and biochemical experiments, under controlled conditions, that we tested in the vineyard and on different genotypes of *Vitis*.

compared to 'Cabernet Sauvignon', where various genes are repressed compared to the susceptible genotype. This approach shows that there is a link between the level of constitutive expression of genes in the different genotypes and their resistance levels to downy and powdery mildews (Fig. 3). After pretreatment with BTH, we mainly observed an increase in the level of gene expression in leaves of susceptible 'Cabernet Sauvignon' and susceptible genotypes, particularly the PR-proteins 1 and 6, two genes of phytoalexins biosynthesis (PAL and STS), glutathione-S-transferase and lipoxygenase genes.

Monitoring gene expression in the vineyard after plant treatments before and after inoculation with downy mildew showed that after three applications and whatever the treatment, the majority of genes coding for PR-Proteins were up-regulated, as were two genes coding for key enzymes of phenylpropanoid biosynthesis, i.e., phenylammonia lyase (PAL) and stilbene synthase (STS). In these two genes, there appears to be a relationship between their level of expression after three treatments and before the epidemic and leaf protection obtained after inoculation with the downy mildew, as shown in Figures 2 and 4. It may be that the more the genes were overexpressed, the more the leaves were protected.

Biochemical Approach

Concerning the biochemical approach, the polyphenol analyses of 'Cabernet Sauvignon' leaves inoculated, revealed that pathogens alone induced accumulation of polyphenols, which was inefficient to inhibit the growth pathogen (Dufour et al., 2013). On the other hand, after treatment with BTH 24 hours before the pathogen inoculation, we noted a 2- to 20-fold an increase in a specific polyphenol, pterostilbene, depending on the time and pathogen considered (Table 3).

Similarly, the amounts of pterostilbene found in the leaves of different genotypes corroborates previous findings, i.e., that the level of protection to downy mildew is related to the amount of pterostilbene present either constitutively with resistant genotypes containing 2- to 5-fold more pterostilbene or in connection with defenses after BTH stimulation, where we observed an increase in pterostilbene in susceptible or partially resistant (RG1) genotypes. In this case, the level of protection of the different genotypes was correlated with the amount of pterostilbene present in the leaves (Fig. 5).

In the vineyard, the qualitative and quantitative analyses of polyphenols showed that the profiles of polyphenols potentially involved in grapevine defense were very variable quantitatively. However, no qualitative differences were observed. For example, just before inoculation and after four treatments, the quantities of resveratrol and piceid (two grapevine phytoalexins) were inversely proportional to the pterostilbene content and were correlated with the overall level of berry protection (Fig. 6).

Forty-eight hours after inoculation, the quantities of two other compounds, the epsilon viniferin and an unidentified compound (A) seemed to correlate negatively for viniferin with the effectiveness of grapevine defenses on grape berries, and positively for compound A (Fig. 7).

CONCLUSIONS

To conclude, the "BioMolChem" approach is functional in vitro and in natura and can be used to assess the effectiveness of plant defense stimulation or the resistance level of different *Vitis* genotypes.

The molecular and biochemical markers used here show that it is possible to correlate the gene expression patterns and the quantities of pterostilbene with the protection levels of plants.

Some PR-proteins are markers of the induction of defenses but not markers of protection such as PR1-PR10. The best markers seem to be PR-2 and PR-3 for plant defense efficacy and PAL and STS for protection in the vineyard. Phytoalexins such as resveratrol and piceid are not good markers of protection but can be used to mark elicitation. Conversely, pterostilbene is a good marker of protection both, in vitro and in

natura, where it and an unidentified polyphenol could be markers of the effectiveness of grapevine defense against downy mildew.

We also show that the effect of protection on the foliage and on clusters depends on the stimulator under consideration, and that the variability of pathogens may also play an important role.

Finally, we now have tools to study the behavior of the grapevine under various conditions of elicitation or infection. These will prove very useful in implementing new control strategies in a highly evolving agrosystem.

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Literature Cited

Belhadj, A., Telef, N., Cluzet, S., Bouscaut, J., Corio-Costet, M.F. and Merillon, J.M. 2008. Ethephon elicits protection against *Erysiphe necator* in grapevine. J. Agr. Food Chem. 56:5781-5787.

Corio-Costet, M.F., Dufour, M.C., Cigna, J., Abadie, P. and Chen, W.J. 2011. Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. Eur. J. Plant Pathol. 129:315-329.

Debieu, D., Corio-Costet, M.F., Steva, H., Malosse, C. and Leroux, P. 1995. Sterol composition of the wine powdery mildew fungus: *Uncinula necator* sensitive or resistant strains to the sterol biosynthesis inhibitor: triadimenol. Phytochemistry 39:293-300.

Dufour, M.C. 2011. Study of the effectiveness of different genotypes of *Vitis vinifera* defenses induced by elicitation face to the genetic diversity of pathogens (*Plasmopara viticola* and *Erysiphe necator*): from gene to the field. Ph.D. dissertation, Bordeaux University, N° 1847, 335p.

Dufour, M.C. and Corio-Costet, M.F. 2013. Variability in the sensitivity of biotrophic grapevine pathogens (*Erysiphe necator* and *Plasmopara viticola*) to acibenzolar-S methyl and two phosphonates. Eur. J. Plant Pathol. DOI 10.1007/s10658-012-0159-2. (in press).

Dufour, M.C., Fontaine, S., Montarry, J. and Corio-Costet, M.F. 2011. Assessment of fungicide resistance and pathogen diversity in *Erysiphe necator* using quantitative real-time PCR assays. Pest Manag. Sci. 67:60-69.

Dufour, M.C., Lambert, C., Bouscaut, J., Mérillon, J.M. and Corio-Costet, M.F. 2013. Benzothiadiazole-primed defense responses and enhanced differential expression of defense genes in *Vitis vinifera* infected with biotrophic pathogens (*Erysiphe necator* and *Plasmopara viticola*). Plant Pathol. Accepted 2nd May 2012, DOI: 10.1111/j.1365.3059.2012.02628.x. (in press).

Fung, R.W.M., Gonzalo, M., Fekete, C., Kovacs, L.G., He, Y., Marsh, E., McIntyre, L.M., Schachtman, D.P. and Qiu, W.P. 2008. Powdery mildew induces defense-oriented reprogramming of the transcriptome in a susceptible but not in a resistant grapevine. Plant Physiol. 146:236-249.

Tally, A., Oostendorp, M., Lawton, K., Staub, T. and Bassi, B. 1999. Commercial development of elicitors of induced resistance to pathogens. p.357-369. In: A.A. Agarwal, S. Tuzun and E. Bent (eds.), Induced Plant Defences against Pathogens and Herbivores, APS Press, St. Paul. p.357-369.

Walters, D., Newton, A. and Lyon, G. 2007. Induced resistance for plant defence: a sustainable approach to crop protection. Blackwell, Oxford, UL. p.258.

Tables

Table 1. Effective concentrations inhibiting growth at 50% (EC_{50}) obtained from dose response curves.

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

* Asterisks represent the significantly different values at the threshold of 0.05% (Dufour and Corio-Costet, 2013).

Table 2. Efficacy of BTH treatment at 1.9 mM, 24 hours before inoculation, expressed as growth of downy mildew isolates or powdery mildew group A or group B isolates on 'Cabernet Sauvignon' and genotypes arising from crossing between *Muscadinia rotundifolia* and *Vitis vinifera* (SG: susceptible genotypes, RG1: resistant genotype I, RG2: resistant genotype 2). Source: Dufour, 2011.

Isolates	Growth of pathogen (%±SEM)			
	CS	SG	RG1	RG2
P. viticola	40.0±8.5	27±11.7	3.1±1.4	0±0.0
E. necator - Group A	0.5±0.3	2±1.3	27±9.3	0±0.0
E. necator - Group B	28±7.8	1.5±0.7	5.3±2.1	0±0.0

Table 3. HPLC quantification of pterostilbene in *Vitis vinifera* (L.) leaves in response to BTH treatment (1.90 mM), with or without pathogen inoculations. Source: Dufour et al., 2013.

	Pterostilbene content (µg/g of dry weight)			
	T0[1]	24 hpi	48 hpi	72 hpi
Control	5.11	0	2.6	2.2
BTH (1.9 mM)	11*	8.24*	5.01	10.14*
E. necator	-	0	0	0
P. viticola	-	4.03	2.17	2.15
P. viticola + BTH	-	9	6.11	7.83*
E. necator + BTH	-	11.1*	16.89*	21*

[1] Before inoculation.
* Asterisks represent significantly different values at threshold of 0.05%.
hpi: hour after inoculation.

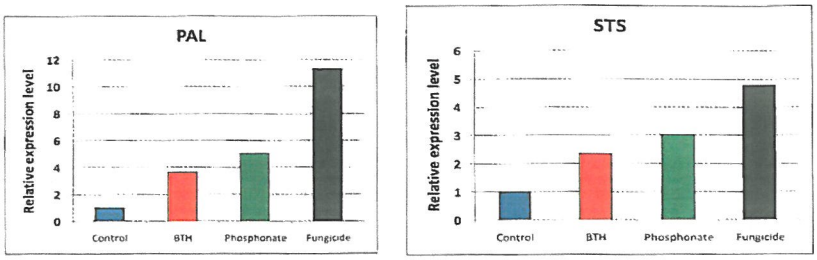

Fig. 4. Relative expression level of phenylammonialyase (PAL) and stilbene synthase (STS), two genes involved in polyphenol biosynthesis in 'Cabernet Sauvignon' leaves, after three treatments in vineyard and before downy mildew inoculation. Source: Dufour, 2011.

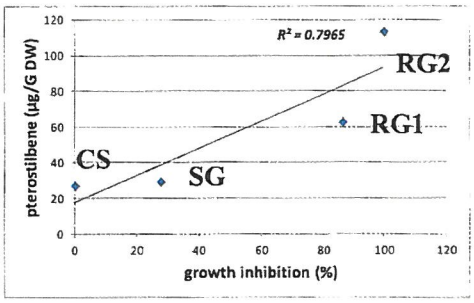

Fig. 5. Correlation between pterostilbene content in different varieties or genotypes and the growth inhibition of *P. viticola* on leaves (in the following order; CS, SG, RG1, RG2).

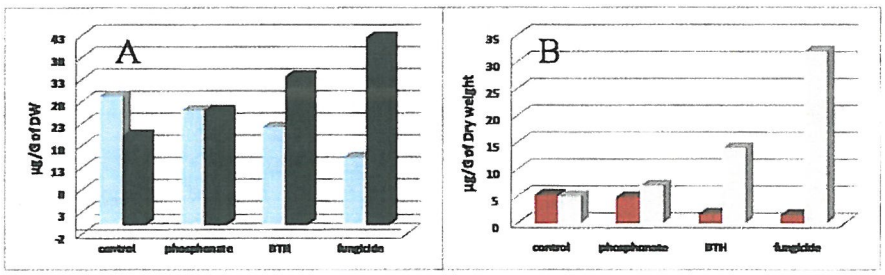

Fig. 6. Stilbene contents in CS leaves in vineyard. (A) pterostilbene (dark blue) and resveratrol + piceid (pale blue) contents in leaves after three BTH treatments and before inoculation. Epsilon viniferin (red) and compound A (grey) contents in leaves after three BTH treatments and 48 hpi after inoculation. Source: Dufour, 2011.

Figures

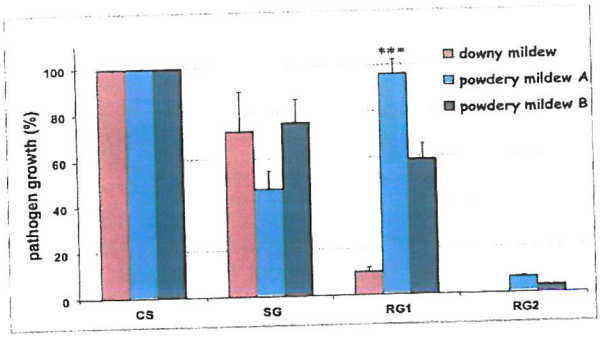

Fig. 1. Growth of downy mildew isolates (pink) or powdery mildew group A (pale blue) or group B isolates (dark blue) on 'Cabernet Sauvignon' and genotypes arising from crossing between *Muscadinia rotundifolia* and *Vitis vinifera* (SG: susceptible genotypes, RG1: resistant genotype I, RG2: resistant genotype 2). Source: Dufour, 2011.

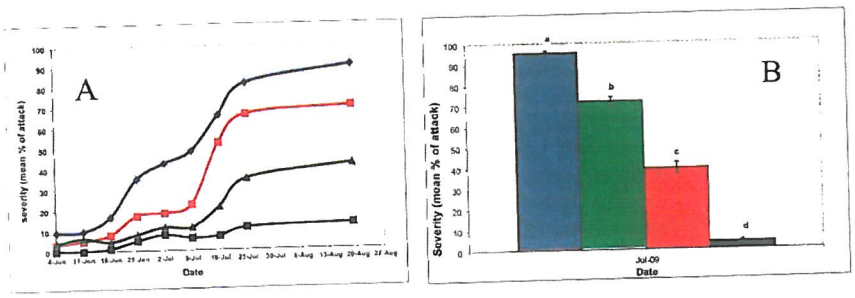

Fig. 2. Efficacy of BTH (red), phosphonate (green) and fungicide treatments (black) on leaf (A) and grape (B) severities in vineyard on CS cultivar. Untreated plots (blue) Source: Dufour, 2011.

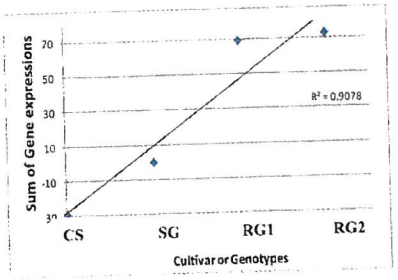

Fig. 3. Correlation between level of constitutive expression of defense genes (20) of grapevine and level of protection against downy mildew of different genotypes (CS=0; GS =1; RG1=2, RG2=3).