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Interactions between *Plasmopara viticola* infection and stilbene synthesis in leaves and berries of ten 'Cabernet Sauvignon' clones

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Summary

Combining the knowledge that *Plasmopara viticola* causes considerable damages to *Vitis vinifera* L. worldwide production and that stilbenes have a regulatory influence on *Plasmopara viticola* - grapevine interaction, this study compares infection time course and stilbenes production in leaves and berries of ten different clones of 'Cabernet Sauvignon'. Following increasing infection rates, different stilbenes were produced and accumulated in leaves and berries of the same clone. Higher absolute values were found in leaves, where *trans*-resveratrol reaches up to 8 µg g^{-1} fw and ε-viniferin up to 30 µg g^{-1} fw while in berries, the values accumulated only up to a maximum of 3 µg g^{-1} fw of trans-piceid and 1,5 µg g^{-1} fw ε-viniferin.

Introduction

Vitis vinifera L. production is highly affected during its life cycle by *Plasmopara viticola* Berk et Curtis, the causal agent of downy mildew. The *Plasmopara viticola* - grapevine interaction has been proven to be affected by the presence of stilbenes (Dercks and Creasy 1989; Pezet *et al.* 2004 a) which are phytoalexins particularly known due to their recognized health related influence. The role of stilbenes in fungus/grapevine interaction can be shortly described for susceptible plants as a rapid glycosylation of resveratrol into the less toxic compound piceid and for resistant plants as a quick oxidization of resveratrol to very fungitoxic viniferins (Pezet *et al.* 2004 b).

Despite a general high susceptibility in *V. Vinifera,* comparisons among cultivars revealed differences in susceptibility (Boso and Kassemeyer 2008). In modern viticulture, great importance is given to the intrinsic characteristics that can be expressed by a variety, not only in terms of viticultural features but also in productive/enological terms which will in turn influence the quality characteristics of the wine produced. As a result, the presence of different clones of a single variety within a vineyard has become common practice and international varieties already have a myriad of clones. Within this view, in this work, both leaves and berries of each singular clone, infected with *Plasmopara viticola* were studied and their capacity for stilbenes production and accumulation over time in different organs was analyzed.

Material and Methods

P l a n t m a t e r i a l : Ten clones of 'Cabernet Sauvignon': clone ISV105, Argentina; clone ISV117, Chile; clones 191, 341, 338, 169 and 685, France; clone R5, Italy; clonesVCR8 and ISV2, USA; were planted in 50 L pots and grown outside, under a hail-protective net, with drip-irrigation from the beginning of the vegetative growth until harvest in order to keep the soil near field capacity. The vines were Guyot trained with 9 buds/vine.

The 4th or the 5th leaf from the shoot tip was harvested before flowering (stage 15 of the Eichhorn and Lorenz stages) then washed with 2 % NaOCl solution for 5 min and rinsed in tap water, and small leaf discs of about 1.8 cm Ø were made. A number of 3 leaf discs were used for each replicate and for every sampling time of each clone the average of 3 replicates is used as final value. The fresh weight of the leaf discs was recorded. Inoculation per immersion with *Plasmopara viticola* was performed, 25,000 sporangia/mL, and the disks were placed in petri dishes with filter paper and water. Visual symptoms and stilbene production were then evaluated in all clones at specific times, just before inoculation, 2 days-post inoculation (dpi), 3 dpi, 5 dpi, 6 dpi, 7 dpi, 8 dpi. Uninfected leaf discs were kept as control. The berries were harvested ten days after fruit set, washed with 2 % NaOCl solution for 5 min and rinsed in tap water. Clusters were cut in smaller pieces, including always berries, pedicels and rachis. Each sample was placed in petri dishes filled with an agar solution to prevent the small bunch parts from dying. A number of 3 replicates consisting each one in a cluster part with at least 10 small berries were used for every sampling time of each clone, the average of 3 replicates is used as final value. The fresh weight of the samples was recorded. The berries were spray inoculated with *Plasmopara viticola,* 25,000 sporangia/mL, and kept in petri dishes with agar. Visual symptoms and stilbene concentrations were controlled just before inoculation, 1 dpi, 3 dpi, 12 dpi and 19 dpi. Uninfected berries were kept as control.

All boxes for leaves and berries were kept in a climatic chamber under controlled humidity (100 %), temperature (20 ± 1°C) and light (12 h/d).

V i s u a l s y m p t o m s e v a l u a t i o n : At each sampling time the percentage of sporulation at the surface of each leaf disc was estimated. Although cluster parts were kept, only the percentage of sporulation present in the surface of each berry was estimated.

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S t i l b e n e e x t r a c t i o n : Leaf disc stilbenes as well as berry stilbenes were extracted and HPLC analyzed according to Bavaresco *et al.* (1997).

S t a n d a r d s : The *trans*-resveratrol (trans-3,4',5-hydroxystilbene) and piceatannol purchased from Sigma (St. Louis, USA) were used as standards; *cis*-resveratrol was prepared from the standard of *trans*-resveratrol by photoisomerization. ε-viniferin (dimer of trans-resveratrol) was purchased from CTChrom (Marly, CH). *Trans*-piceid standard was also utilized. The purity of each stilbene was controlled by HPLC, and the identity was confirmed.

H P L C c o n d i t i o n s : An Agilent HP 1100 series (Waldbronn, Germany) with an autosampler and diode array detector (DAD) set at 306 and 325 nm. A 250 × 4.6 mm i.d., 5µm, a C 18 Supelco Supelcosil ABZ plus column was used for leaf extracts, eluting with a gradient of methanol (A) and 0.01 M potassium phosphate monobasic adjusted to pH 2.5 with phosphoric acid (B). The gradient was 40 to 85 % of A at a flow rate of 1.0 mL min^{-1}. The injection volume was 50 µL. A Phenomenex Gemini 3 µm C18 110A column, 100 x 4.6mm was used for berry extracts, with a gradient of 40 to 85 % of A at a flow rate of 0.6 mL min^{-1}.

S t i l b e n e s q u a n t i f i c a t i o n : Amounts of stilbene standards between 1 and 500 ng were injected. Quantification was on the bases of peak areas using the PC software.

S t a t i s t i c a l a n a l y s i s : Data analysis was conducted using SPSS version 15.0.1, licensed for USCS Piacenza. First Fisher's F was determined and when appropriate the Waller-Duncan test was used.

Results

Statistical differences were found amongst the leaf discs of the studied clones in all measured samples concerning visual symptoms. In the last sampling (Figure A), clone Vcr8 reached over 10 % of sporulation and clone R5 presented the highest values, over 25 % of infected surface. Also in the infected berries several statistically significant differences arose amongst the clones and at T4 (Figure B), the last sampling, most clones reached values closer to 10 % while clone 338 reached values over 20 %.

Stilbene measurements in the leaf discs lead only to the detection of *trans*-resveratrol and ε-viniferin after T1, at T0 no stilbenes were detected. The analysis of *trans*-resveratrol accumulation in the *Plasmopara viticola* infected leaf discs (Table) shows that there were only statistically significant differences at T2 and T3. Comparison of ε-viniferin accumulation over time (Table) showed significant differences at T3 and T5, while its presence could not be traced in some clones at T1.

In berries (Table), *trans*-piceid and ε-viniferin were found and their presence could already be detected at T0. Statistical differences in *trans*-piceid accumulation values were found between the clones at all the sampling times. No statistically significant differences could be found at any of the sampling times between the studied clones for ε-viniferin (Table), for most clones values can only be traced in 2 sampling times. In the present study absolute stilbene values are definitely higher in the leaves where *trans*-resveratrol reached up to 8 µg·g^{-1} fw and ε-viniferin up to 30

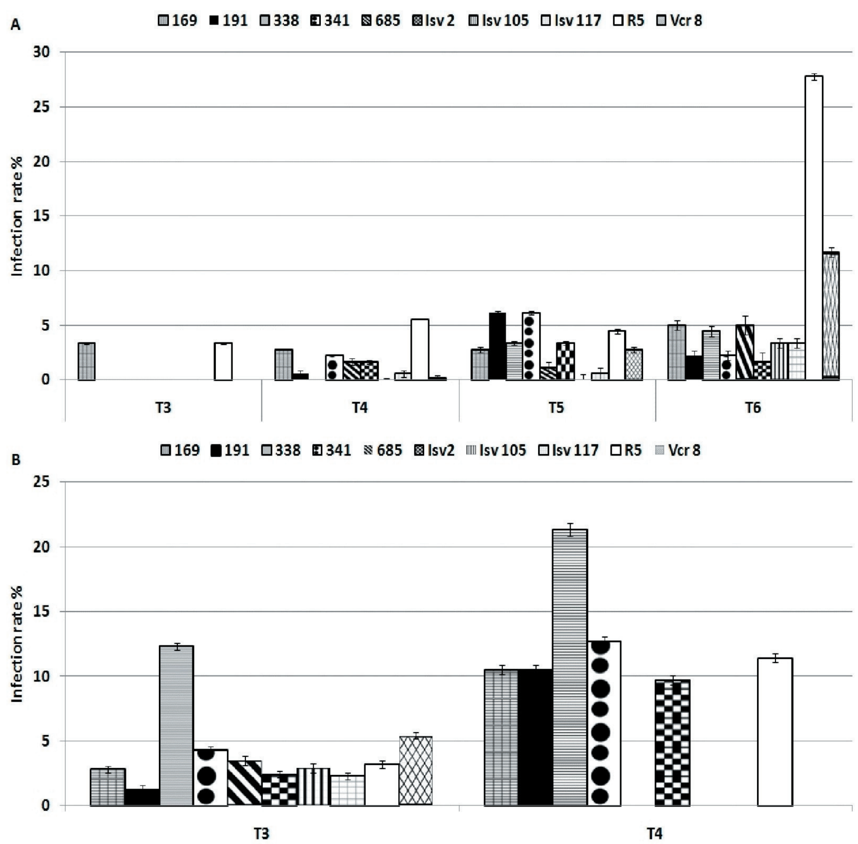

Figure: Infection rates in *Plasmopara viticola* infected leaf discs (**A**) and berries (**B**) of 10 different clones of 'Cabernet Sauvignon': clones 169, 191, 338, 341, 685, ISV2, ISV105, ISV117, R5 and VCR8.

T a b l e

Comparison of stilbene values (µg·g^{-1} fw) detected per clone after infection with *Plasmopara viticola: trans*-resveratrol and ε-viniferin in leaf discs and *trans*-piceid and ε-viniferin in berries. Values with the same letter are not significantly different (α < 0.05 WD Test) -: no stilbenes were detected in at least 2 of the replicates

		T0		T1		T2		T3		T4		T5		T6	
	Clone	*t*-resv	ε-vinif	*t*-resv	ε-vinif	*t*-resv	ε-vinif	*t*-resv	ε-vinif	*t*-resv	ε-vinif	*t*-resv	ε-vinif	*t*-resv	ε-vinif
Leaves	169	-	-	2,756	-	2,069 a	5,571	1,907 a	12,926 ab	1,882	6,14	1,272	5,799 abc	2,165	8,348
	191	-	-	2,596	3,388	2,700 a	2,23	3,163 a	2,504 a	2,131	18,19	1,892	3,184 a	2,722	7,318
	338	-	-	2,879	3,147	8,039 b	5,864	2,235 a	2,787 a	2,135	9,057	-	5,979 abc	3,325	11,43
	341	-	-	4,519	3,313	3,039 a	2,307	3,657 a	4,194 ab	1,435	15,72	-	6,602 abc	-	-
	685	-	-	6,301	-	4,025 ab	7,143	3,599 a	6,754 ab	4,392	31,00	3,593	14,614 bc	4,160	5,427
	Isv 2	-	-	3,613	3,949	6,024 ab	10,53	7,476 b	4,069 ab	2,770	22,49	1,077	12,349 abc	1,981	6,715
	Isv 105	-	-	3,686	-	2,105 a	6,566	3,137 a	22,651 b	-	13,49	1,89	8,395 abc	1,885	19,86
	Isv 117	-	-	3,557	-	5,610 ab	9,083	-	3,462 ab	3,357	26,14	1,668	4,902 ab	1,459	5,894
	R5	-	-	1,382	-	2,403 a	4,653	4,600 ab	7,005 ab	2,949	5,231	2,352	6,927 abc	2,491	4,21
	Vcr 8	-	-	3,159	4,356	5,133 ab	10,48	7,218 b	20,172 ab	2,022	15,86	2,301	16,114 c	3,938	6,444
	F			0,967	0,078	2,521	1,144	4,067	2,093	1,544	1,184	1,597	2,500	1,099	0,801
	Sig.			n.s.	n.s.	*	n.s.	**	*	n.s.	n.s.	n.s.	*	n.s.	n.s.

		T0		T1		T2		T3		T4	
	clone	*t*-piceid	ε-vinif	*t*-piceid	ε-vinif	*t*-piceid	ε-vinif	*t*-piceid	ε-vinif	*t*-piceid	ε-vinif
Berries	169	2,732 b	-	1,435 ab	-	1,682 d	-	1,544 b	0,465	1,512 b	0,872
	191	0,921 a	-	2,108 b	-	1,991 d	-	1,628 b	1,036	1,771 b	1,133
	338	-	-	1,467 ab	-	0,170 a	-	0,396 a	-	1,124 ab	1,369
	341	1,563 ab	-	0,460 a	-	0,387 ab	-	0,250 a	0,710	-	-
	685	1,049 ab	0,057	-	-	0,538 abc	-	0,730 ab	0,551	-	-
	Isv 2	0,777 a	-	-	-	-	-	0,753 ab	-	1,099 ab	3,327
	Isv 105	1,555 ab	-	2,003 b	-	0,829 bc	-	1,782 b	0,435	-	-
	Isv 117	1,148 ab	0,064	0,796 ab	-	0,715 abc	-	1,147 ab	-	-	-
	R5	0,871 a	-	1,186 ab	-	1,028 c	-	0,355 a	0,781	0,289 a	1,552
	Vcr 8	0,652 a	0,037	0,882 ab	-	0,903 bc	-	1,041 ab	0,435	-	-
	F	2,061	1,288	2,561		10,764		3,290	2,220	3,817	0,591
	Sig.	*	n.s.	*		***		*	n.s.	*	n.s.

μg·g^{-1} fw (clones 338 and 685, respectively) while in berries, the values rose only up to 3 μg·g^{-1} fw of *trans*-piceid and up to 1.5 μg·g^{-1} fw of ε-viniferin, clones 169 and Isv2 respectively.

Discussion

It is important to notice that in all berries, stilbenes were already present in T0, before inoculation, while in leaves no stilbenes could be measured, implying that the presence of a pathogen could be necessary for the activation of the phytoalexins as a defence system while a basic stilbene pool for defence seems to be present in small growing berries.

ε-viniferin is the only common compound found in both leaves and berries and apart from this compound, *trans*-resveratrol could be found in the leaves and *trans*-piceid in the berries of the 10 clones. Given the differences found in literature concerning the differences in fungitoxicity found among stilbenes (Pezet *et al.* 2004 b, Bavaresco *et al.* 2009), their presence in a certain organ can be linked to a decrease or increase of resistance. Studies conducted using powdery mildew in diverse varieties commonly produced in France (Boubals 1961) support the finding that differences could be found in the resistance of different organs of the same variety, furthermore resveratrol in the grapevine appears as organ-specific and tissue-specific (Wang *et al.* 2010), in berries for instance, the type of stilbenes detected and their resistance related nature was proven to be genotype dependent (Gatto *et al.* 2008).

The presence of both resveratrol and ε-viniferin in leaves is supported by the trial of Jean-Denis *et al.* (2006) while in berries, the presence of *trans*-piceid and ε-viniferin can be supported by other trials (Pezet *et al.* 2004) that also showed that in susceptible varieties, resveratrol seems to be glycosylated into piceid and it is possible that the initial values of resveratrol are too low to allow the production of viniferins, in fact the viniferins tend to be detected only some time after inculation which can be due to the necessary presence of peroxidade synthase whose activation is slower than that of STS. The differences found between infection rates and stilbenes synthesis within the 10 clones of 'Cabernet Sauvignon' in leaves and berries can be justified not by varietal differences but by the genotypical differences that can be found within the clones, a result of the genetic variation that characterises them.

Conclusions

Through the analysis of 10 different clones of 'Cabernet Sauvignon' it was possible to determine that *Plasmopara viticola* infected leaves and berries of the same vine synthesise different stilbenes, differences which may explain different resistance levels in the different organs.

In berries, stilbenes could be traced before inoculation despite the fact that leaves were able to accumulate higher total values of stilbenes.

All differences found between infection rate and stilbenes synthesised and accumulated appeared as clone dependent whether considering leaves or berries.

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