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Elicitation and accumulation of stilbene phytoalexins in grapevine berries infected by *Botrytis cinerea*[*)]

by

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S u m m a r y : At three developmental stages berries of field-grown Castor (interspecific crossing) and Huxelrebe (*V. vinifera* L. crossing) were *in vitro* inoculated with two strains of *Botrytis cinerea* Pers. to investigate the response of berries to fungal infection with respect to the time course of phytoalexin (*trans*-resveratrol, ε-viniferin and pterostilbene) accumulation and visual disease symptoms. In infected berries the amounts of ε-viniferin dominated over pterostilbene. The stilbene phytoalexin content decreased during berry development and sugar accumulation. Grape varieties reacted differently to *B. cinerea* strains with regard to stilbene response and visual symptoms. Mechanical damage of the berry skin induced uninfected berries to synthesize low amounts of phytoalexins. It can be assumed that after extraction and degradation ε-viniferin of mature berries is a source of resveratrol in wine.

K e y w o r d s : grapevine, berry, trans-resveratrol, ε-viniferin, pterostilbene, Botrytis cinerea.

Introduction

Botrytis cinerea Pers., anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel, the causal agent of grey mould, seriously reduces grape yield and quality by converting sugar into glycerol and gluconic acid and by producing enzymes catalysing oxidation of phenolic compounds such as stilbene phytoalexins. Rapid accumulation of phytoalexin (*trans*-resveratrol and ε-viniferin) has been associated with the resistance of grapevines to *B. cinerea* (Langcake and Mc Carthy 1979; Blaich *et al.* 1982; Jeandet and Bessis 1989), which is normally observed in American species and interspecific crossings. Phytoalexins represent only one mechanism involved in resistance (Fregoni 1983; Faretra and Mayer 1992), others are thickness and structure of cuticular waxes (Rosenquist and Morrison 1989; Comménil *et al.* 1996), low number of cuticle perforations (Bessis 1972 a; Blaich *et al.* 1984), polyphenols and glycolic acid (Jeandet and Bessis 1989; Pezet and Pont 1988 a), and probably PR proteins (Renault *et al.* 1996). On the other hand, phytoalexin synthesis of susceptible plants is reduced, and does not reach high concentrations due to both, the genetic pattern traits of the vines and the activity of fungal laccase-like enzymes (Pezet *et al.* 1991; Jeandet *et al.* 1993). The subject of stilbene phytoalexins and disease resistance has been recently reviewed by Dercks *et al.* (1995).

Trans- and *cis*-isomers of resveratrol are also present in wine (Siemann and Creasy 1992), and resveratrol is supposed to be the active principle of red wines reducing heart deseases (Seigneur *et al.* 1990; Renaud and De Lorgeril 1992).

The aim of this paper was to study the role of stilbene phytoalexins in the *B. cinerea* grapevine interaction, and their significance for the origin of resveratrol in wine.

Materials and methods

P l a n t m a t e r i a l : 15-year-old plants of Castor (Oberlin 595 F1 × Foster's White Seedling) and the *V. vinifera* L. variety Huxelrebe (Weißer Gutedel × Courtillier musqué), grown in an experimental vineyard near Piacenza (northern Italy), were used for the trial. Castor was originally supplied by the Institut für Rebenzüchtung Geilweilerhof, Siebeldingen/Germany, while Huxelrebe was obtained from the LA für Rebenzüchtung Alzey, Germany. Castor is considered to be resistant to *B. cinerea* under German conditions (Alleweldt 1980), whilst Huxelrebe is susceptible (Hillebrand *et al.* 1984). These two varieties were chosen due to their different disease resistance and due to their phenological similarity in the Piacenza area (Bavaresco and Boselli 1986). Grape berries from 20 clusters (chosen at random from 5 plants of each variety) were sampled about 25 d after fruit set, at veraison and during ripening, i.e. in the first, second and

T a b l e 1

Average berry weight, soluble solids and pH of berry juice of Castor and Huxelrebe during berry development

		Average berry weight (g)	Soluble solids (°Brix)	pH
Castor	25 d after fruit set	1.1	4.1	2.72
	at veraison	1.4	7.5	2.42
	during ripening	2.7	19.4	3.39
Huxelrebe	25 d after fruit set	1.0	4.0	2.66
	at veraison	2.1	10.8	2.55
	during ripening	2.9	18.0	3.24

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third stage of berry growth. After analysis of berry weight, soluble solids and pH (Tab. 1) the berries were artificially inoculated with *B. cinerea*.

Preparation of fungal inoculum: Two *B. cinerea* strains (SAR 2116 and SAR 2228) supplied by F. FARETRA (Dept. Plant Protection, University of Bari) were obtained from crossings between monoascosporic isolates collected from grapevine, rose and carnation in the Apulia region (southern Italy). Due to observations of leaf and berry symptoms at several grape varieties at veraison and maturity, SAR 2116 is considered to be of low and SAR 2228 to be of high virulence.

Fungal colonies were transferred and cultured in Petri dishes containing 20 ml of 1.5 % Czapek (Dox) Agar (CZ), according to SMITH and ONIONS (1983); they were incubated at 25±1°C and a 12 h photoperiod for 2 weeks. Conidial suspension was prepared from 14-day-old cultures by flooding the Petri dishes with a sterile solution (0.01 % Tween 20) and scraping the agar surface to dislodge conidia. The suspension was filtered through a double layer cheese cloth and conidia concentration was measured using a Bürker chamber and set to 5×10^5 conidia·ml^{-1}.

Berry inoculation: Berries with pedicels were carefully excised from the rachis in order to preserve their integrity and washed in running water for 2 h. After surface sterilization (2 % NaOCl, 5 min) and rinsing twice in sterile water, 48 berries were placed on a sterile rectangular metal net (6 rows of 8 berries each), with square links, a small sterile aluminium basin and blotting paper on the bottom. Berry skins were pricked by a sterile needle close to the pedicel and a drop of inoculum (20 µl) was placed upon. Control (uninoculated) berries were treated with a suspension without conidia. The basins were wrapped up with a plastic bag and placed inside a growth chamber (25±1 °C, 16 h of light per day, light intensity: 40 µmol m^{-2} s^{-1}).

Extraction and identification of stilbene phytoalexins: 0.5, 1, 2, 4, 8 and 16 d after inoculation berries were analysed. All analytic values are means of 3 replicates of 8 berries each. To analyse the stilbene phytoalexins *trans*-resveratrol, ε-viniferin and pterostilbene berries were ground in a mortar with 30 ml of 95 % methanol and vigorously shaken for 20 min. Seeds of the second (veraison) and third (maturity) sampling were discarded before maceration in order to avoid extraction of constitutive resveratrol. A filtration by GF/A Whatman filters followed, the liquid was evaporated *in vacuo* at 40 °C and the water fraction was extracted twice with 5 ml ethylacetate and 5 ml $NaHCO_3$ (5 %), by phase partitioning. The organic phase was evaporated to dryness and stilbene compounds were recovered by 2 ml plus 2 × 1 ml methanol (100 %) and stored in adiactinic glass vials at 4 °C. Just before stilbene analysis the samples were evaporated by a stream of dry N_2 and dissolved in 2 ml of methanol 50 %. Stilbene analyses were done by HPLC as de-

Table 2

Effect of grape variety, berry development, fungus strain and incubation time on berry phytoalexin concentration and infection index

		Trans-resveratrol µg g^{-1} FW	ε-Viniferin µg g^{-1} FW	Pterostilbene µg g^{-1} FW	Infection index %
Grape variety[1])	Castor	5.00	6.64	0.13	5.3
	Huxelrebe	2.11	3.30	0.06	7.1
	$LSD_{0.05}$	1.07	1.84	n.s.	n.s.
Stage of berry development[2])	25 d after fruit set	6.56	10.30	0.24	3.5
	at veraison	3.80	4.06	0.03	4.7
	during ripening	0.30	0.54	0.01	10.4
	$LSD_{0.05}$	1.32	2.26	0.10	6.0
Fungus strain[2])	control	1.64	1.37	0.04	0.5
	SAR 2116	3.79	5.35	0.03	9.5
	SAR 2228	5.24	8.19	0.21	8.7
	$LSD_{0.05}$	1.32	2.26	0.10	7.1
Incubation time[3])	0.5 d	0.96	0.49	0.17	0
	1 d	2.52	1.79	0.25	0
	2 d	6.56	4.23	0.01	0
	4 d	7.31	11.69	0.06	2.7
	8 d	2.54	7.58	0.05	9.4
	16 d	1.44	4.04	0.01	25.2
	$LSD_{0.05}$	1.86	3.19	0.14	4.7

[1-3]) Each stilbene value is the mean of 162 (1), 108 (2) or 54 data (3).

scribed by PEZET *et al.* (1994), utilizing, as standard compounds, *trans*-resveratrol (Sigma), ε-viniferin (provided by G. HOOS, formerly Institut für Rebenzüchtung Geilweilerhof, Siebeldingen/Germany), and pterostilbene (provided by R. PEZET, FARS Changins, Nyon, Switzerland). A liquid chromatograph (Hewlett Packard 1090 L, Waldbronn, Germany) with autosampler (10 µl of injection volume) and DAD (diode array detector, $\lambda = 310$ nm) and a Lichrospher column (100 RP, Merck, 125 x 4 mm, 5 µm particle size) were used. Elution: gradient of methanol (from 20 to 80 % in 27 min) and formic acid (0.24 %); flow rate: 1.0 ml·min $^{-1}$.

Visual infection symptoms: Symptoms were classified according to the following scale: 0 = no symptoms; 1 = partial berry infection; 2 = total infection with aerial mycelium. The infection index (I.I.) was calculated as:

$$I.I. = \left[\left(\sum_{i=0}^{2} n_i \times i\right) / \left(\sum_{i=0}^{2} n_i \times 2\right)\right] \times 100$$

where i = scale rating (0 to 2), n = number of berries in each scale rating.

Statistical analysis: A four-way-ANOVA (considering genotype, berry growth stage, fungal strain, incubation time as main effects) with interactions was utilized, and the means were compared by using the LSD test (5 % level). Data of berry infection rate were submitted to angular transformation.

Results

On the average, berries of the *Botrytis*-resistant variety Castor synthesized more *trans*-resveratrol (5 µg g^{-1} FW) and ε-viniferin (6.6 µg g^{-1} FW) compared to the susceptible variety (2.1 and 3.3 µg g $^{-1}$ FW, respectively). The amount of pterostilbene was much lower than the other stilbenes (no significant difference between the two varieties, Tab. 2). The concentrations of the three compounds decreased from the first to the third stage of berry growth indicating very low levels at ripening.

Also uninoculated berries produced stilbenes (Tab. 2), even though to a much lower extent than the inoculated ones; SAR 2228 elicited a higher stilbene synthesis than SAR 2116.

The *trans*-resveratrol and ε-viniferin accumulation was enhanced up to the fourth day after inoculation and then dropped till day 16. The values of *trans*-resveratrol were higher than those of ε-viniferin until the second day after inoculation, while afterwards more ε-viniferin than *trans*-resveratrol was found. A maximum of 0.25 µg g^{-1} FW of pterostilbene was reached 1 d after inoculation (Tab. 2).

Castor was less infected than Huxelrebe, and the infection index increased with berry development and with incubation time; *B. cinerea* strains induced similar symptoms (Tab. 2).

During the first stage of berry growth, the effect of the SAR 2228 strain on Castor was significant; this variety

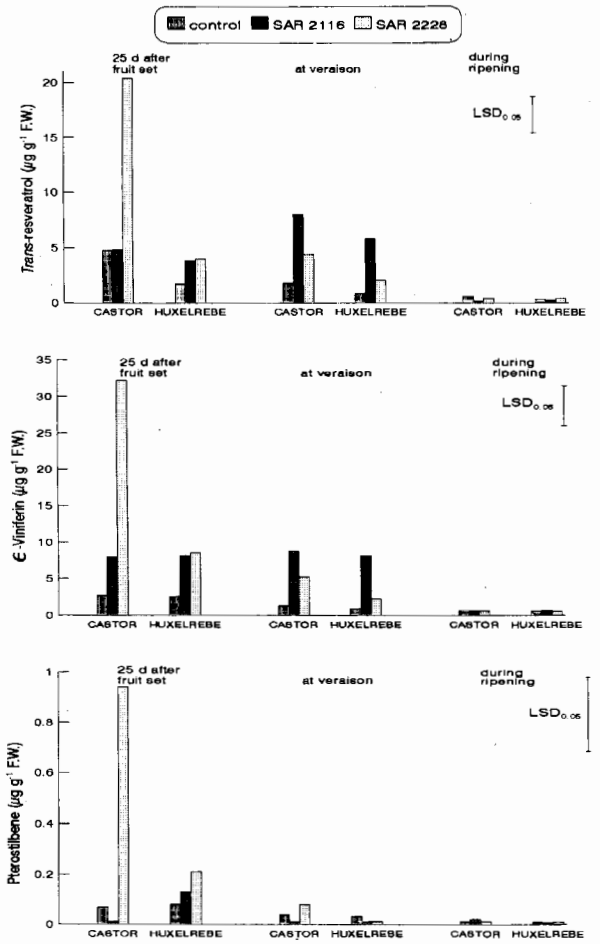

Fig. 1: Concentration of *trans*-resveratrol, ε-viniferin and pterostilbene in berries as a function of grape variety, berry development and fungus strain.

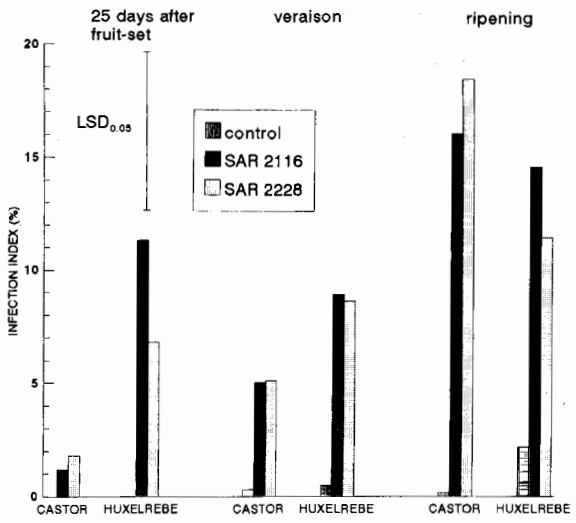

Fig. 2: Berry infection index as a function of grape variety, berry development and fungus strain.

synthesized the highest levels of the three stilbenes, while in Huxelrebe the two *Botrytis* strains elicited the same amount of phytoalexins (Fig. 1). At veraison, on the other hand, the SAR 2116 strain induced higher levels of *trans*-resveratrol and ε-viniferin than SAR 2228 in both varieties; no significant differences were found at ripening.

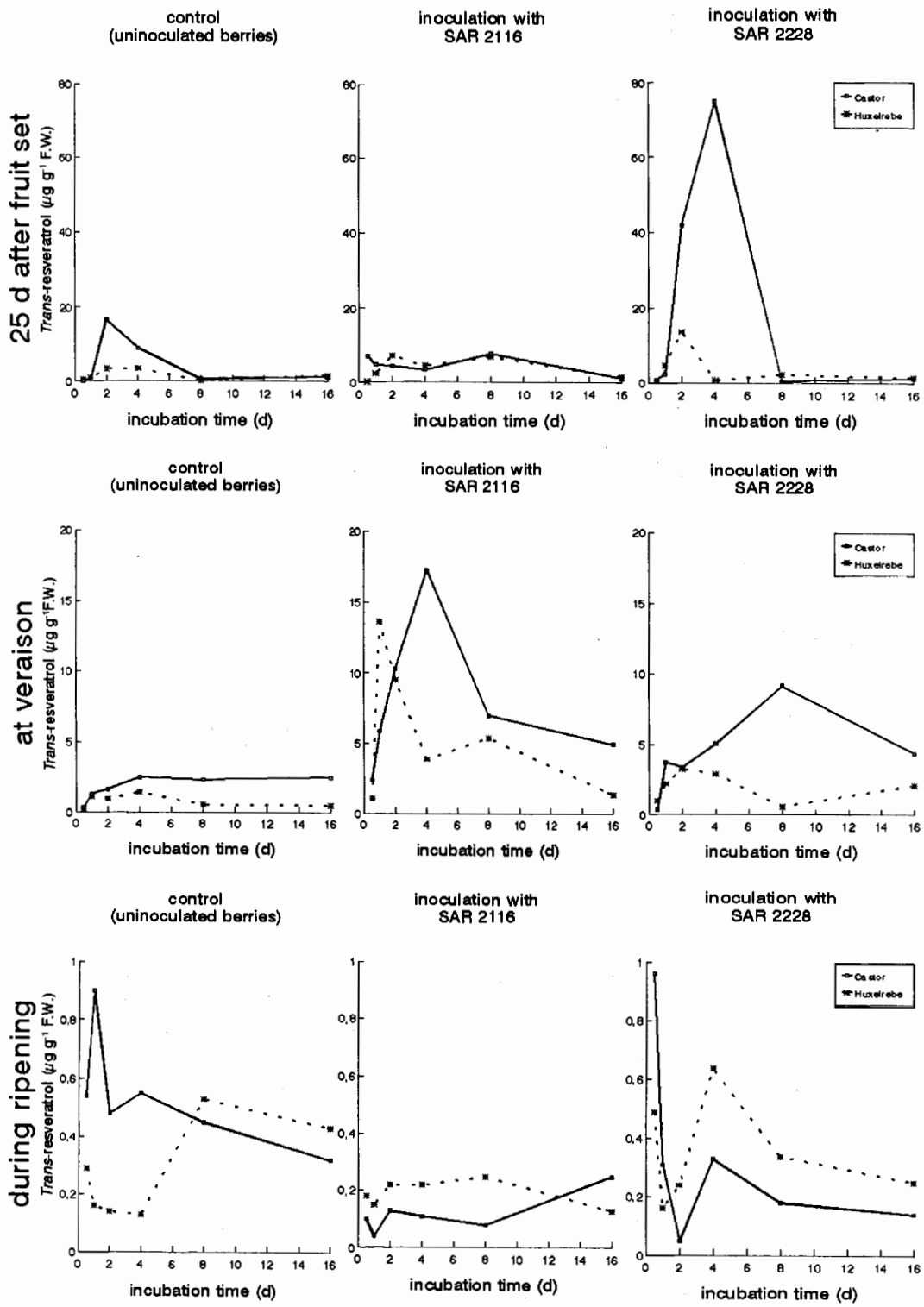

Fig. 3: Time course of *trans*-resveratrol accumulation in berries of two varieties and the effect of fungus strain. Each value is the mean of three replicates.

During the first stage of berry growth no differences of infection symptoms between the two strains were observed with Castor while with Huxelrebe the SAR 2116 strain induced more symptoms than SAR 2228 (Fig. 2). At veraison no infection differences between the two strains were recorded, whereas at maturity the effect was different depending on the variety.

The time course of *trans*-resveratrol and ε-viniferin accumulation differed distinctly depending on the variety, stage of berry development and fungal strain (Figs. 3, 4). At the early stage of berry development and when infection was done with SAR 2228, Castor showed a more rapid response to elicitation than Huxelrebe. It is interesting to note that berries sampled at veraison still had a consistent stilbene content 16 d after inoculation; even at ripening the phytoalexins were not completely degradated 16 d after the treatment.

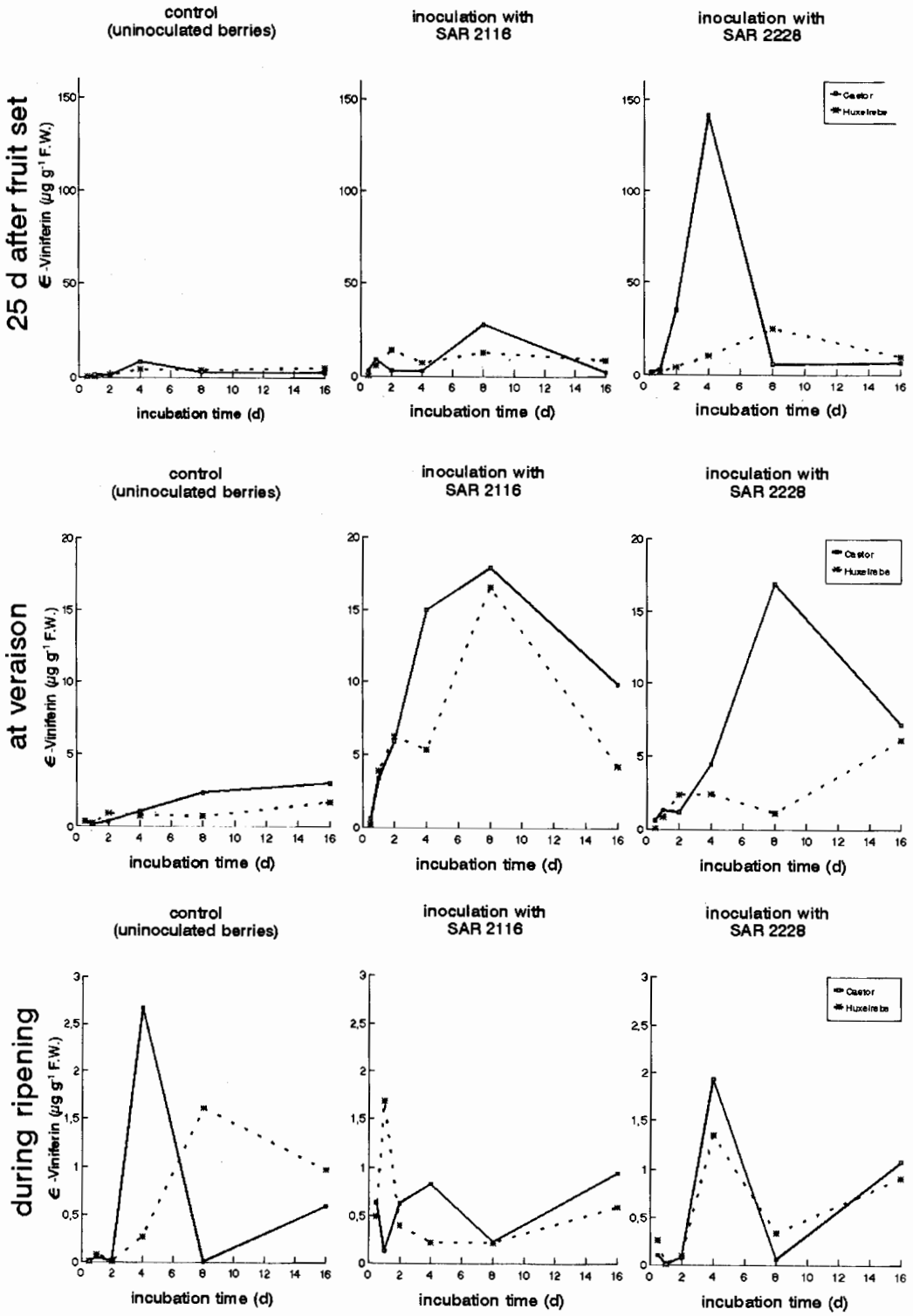

Fig. 4: Time course of ε-viniferin accumulation in berries of two varieties and the effect of fungus strain. Each value is the mean of three replicates.

Discussion

This paper reports for the first time appreciable amounts of *trans*-resveratrol and ε-viniferin in berries of two genotypes which had been infected with two *B. cinerea* strains at different developmental stages. As expected, the interspecific crossing Castor synthesized more stilbenes than the intraspecific crossing Huxelrebe, especially in the early stages of berry development. Evidence has already been obtained for the capability of interspecific varieties and American species to accumulate high stilbene levels in elicited leaves (STEIN and HOOS 1984; STEIN and BLAICH 1985; BAVARESCO and EIBACH 1987; BAVARESCO 1993; BAVARESCO *et al.* 1994), while only few data are available for elicitation in berries (CREASY and COFFEE 1988; JEANDET *et al.* 1991 and 1995 b). The average levels of *trans*-resveratrol were always lower than those reported in the literature for UV-elicited berries of *V. vinifera* cv. Pinot

noir and *V. labrusca* (JEANDET *et al.* 1991). This is likely due to berry colour, since there is some evidence that wines from red varieties have twice as high *trans*-resveratrol concentrations than white ones (JEANDET *et al.* 1995 a). This is not always true, because, according to SOLEAS *et al.* (1995), some white grape varieties had higher *trans*-resveratrol skin concentrations than red varieties.

Quantitatively ε-viniferin seems to be the most important stilbene of *B. cinerea* berry interactions. After the finding of ε-viniferin as new phytoalexin by LANGCAKE and PRYCE (1977) very few data have been obtained on its concentration in berries (LANGCAKE 1981). Like *trans*-resveratrol, the interspecific crossing synthesized more ε-viniferin than the *V. vinifera* cultivar.

Finally, in both varieties the concentration of pterostilbene in the berries was much lower than that of the other stilbenes. This compound is considered to be an important resistance factor of unripe berries to grey mould (PEZET and PONT 1988 a).

The decrease of stilbene concentration from early stages of berry development till ripening confirms data in literature (CREASY and COFFEE 1988; JEANDET *et al.* 1991). The high stilbene level before veraison is considered to be one of the factors explaining the often recorded rate of grey mould resistance (HILL *et al.* 1981). According to JEANDET *et al.* (1995 d) the decreasing stilbene concentration from veraison to maturity could be explained by a competition between chalcone synthase and stilbene synthase, since the former is involved in the flavonoid synthesis, and the latter in the stilbene biochemical pathway. Low stilbene concentrations in *Botrytis*-elicited berries at ripening, could also be due to a detoxification of phytoalexins and/or a laccase-like stilbene oxidase activity, which has been investigated by PEZET *et al.* (1991), JEANDET *et al.* (1993) and SBAGHI *et al.* (1996) (for further discussion see also HOOS and BLAICH 1988).

Even stilbene concentration of uninoculated berries decreased during berry growth, reaching at ripening similar values as *Botrytis*-infected ones. Stilbene production in control berries can be explained by an elicitation due to needle pricking, or by an accidental infection with fungi other than *B. cinerea*, since unelicited berries normally do not produce stilbenes. BESSIS (1972 b) has reported the presence of *B. cinerea* on berries which did not exhibit disease symptoms, thus stilbene synthesis might have been induced (JEANDET *et al.* 1995 c), even though the berries are apparently healthy. According to ECTOR *et al.* (1996) many *Vitis rotundifolia* varieties accumulate resveratrol in unelicited berries while OKUDA and YOKOTSUKA (1996) found very low levels of resveratrol in ripe and apparently healthy berries of *V. vinifera* and interspecific varieties growing in Japan.

The ε-viniferin concentration of both control and inoculated berries at ripening, was higher than *trans*-resveratrol and pterostilbene; this oligo-stilbene is a possible source of resveratrol in wine (GOLDBERG *et al.* 1995).

The effect of the fungal strain on phytoalexin level was evident in the early stage of berry growth in Castor. During ripening, no significant differences between the strains were observed. This may partly be due to changes of virulence of the fungal strains (SBAGHI *et al.* 1996).

The time course of *trans*-resveratrol and ε-viniferin accumulation in berries was similar, with the maximum amounts 4 d after inoculation. The late ε-viniferin data being higher than those of *trans*-resveratrol confirm the role of the latter as precursor of viniferins. On the other hand, pterostilbene showed a more rapid response to elicitation with the highest value 24 h after inoculation. Besides synthesizing higher total amounts of stilbenes, Castor showed also a faster accumulation of *trans*-resveratrol and ε-viniferin than Huxelrebe. The response of ripe berries was very fast with regard to *trans*-resveratrol. No correlations between the time course of the three stilbenes and the infection index was found, except 4, 8, and 16 d after infection, when decreasing stilbene levels were related to increasing rates of infection.

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