Elicitation and accumulation of stilbene phytoalexins in grapevine berries infected by Botrytis cinerea

by

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Summary: 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.

Key words: 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 McCarthy 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; Commenil 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 (Sie mann and Creasy 1992), and resveratrol is supposed to be the active principle of red wines reducing heart diseases (Seigneur et al. 1990; Renaud and De Lorgeril 1992).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Average berry weight (g)</th>
<th>Soluble solids (%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 d after fruit set</td>
<td>1.1</td>
<td>4.1</td>
<td>2.72</td>
</tr>
<tr>
<td>at veraison</td>
<td>1.4</td>
<td>7.5</td>
<td>2.42</td>
</tr>
<tr>
<td>during ripening</td>
<td>2.7</td>
<td>19.4</td>
<td>3.39</td>
</tr>
<tr>
<td>Huxelrebe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 d after fruit set</td>
<td>1.0</td>
<td>4.0</td>
<td>2.66</td>
</tr>
<tr>
<td>at veraison</td>
<td>2.1</td>
<td>10.8</td>
<td>2.55</td>
</tr>
<tr>
<td>during ripening</td>
<td>2.9</td>
<td>18.0</td>
<td>3.24</td>
</tr>
</tbody>
</table>

Materials and methods

Plant material: 15-year-old plants of Castor (Oberlin 595 F1 x Foster’s White Seedling) and the V. vinifera L. variety Huxelrebe (Weißer Gutedel × Courtiller 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 (Bavarese and Bogessi 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 third third of the harvesting period.

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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 Ünions (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 NaHCO₃ (5%), by phase partitioning. The organic phase was evaporated to dryness and stilbene compounds were recovered by 2 ml plus 2x1 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 N₂ 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 variety¹)                |                               |                             |                              |                   |
| Castor                         | 5.00                          | 6.64                        | 0.13                         | 5.3               |
| Huxelrebe                      | 2.11                          | 3.30                        | 0.06                         | 7.1               |
| LSD₀₀₅                         | 1.07                          | 1.84                        | n.s.                         | n.s.              |
| Stage of berry development²)   |                               |                             |                              |                   |
| 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              |
| LSD₀₀₅                         | 1.32                          | 2.26                        | 0.10                         | 6.0               |
| Fungus strain²)               |                               |                             |                              |                   |
| 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               |
| LSD₀₀₅                         | 1.32                          | 2.26                        | 0.10                         | 7.1               |
| Incubation time³)             |                               |                             |                              |                   |
| 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              |
| LSD₀₀₅                         | 1.86                          | 3.19                        | 0.14                         | 4.7               |

¹) 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, λ = 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:

\[
I.I. = \left( \frac{\sum n_i \times i}{\sum n_i \times 2} \right) \times 100
\]

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 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.
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 degraded 16 d after the treatment.

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.
Discussion

This paper reports for the first time appreciable amounts of trans-resveratrol and 6-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 con-
centrations 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 e-viniferin seems to be the most im-
portant stilbene of B. cinerea berry interactions. After the
finding of e-viniferin as new phytoalexin by LANGCAKE and
PRYCE (1977) very few data have been obtained on its con-
centration in berries (LANGCAKE 1981). Like trans-
resveratrol, the interspecific crossing synthesized more
e-viniferin than the V. vinifera cultivar.
Finally, in both varieties the concentration of ptero-
stilbene in the berries was much lower than that of the
other stilbenes. This compound is considered to be an
important 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
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 concentra-
tion from veraison to maturity could be explained by a
competition between chalcone synthase and stilbene
synthase, since the former is involved in the flavonoid syn-
Low stilbene concentrations in Botrytis-elicited berries at
ripening, could also be due to a detoxification of phyto-
alexins and/or a laccase-like stilbene oxidase activity, which
has been investigated by PEZET et al. (1991), JEANDET et al.
(1993) and SABATH 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 simi-
lar 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 pres-
ence 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 appar-
ently 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. vinvifera and interspecific varieties growing in
Japan.

The e-viniferin concentration of both control and in-
oculated berries at ripening, was higher than trans-
resveratrol and pterostilbene; this oligo-stilbene is a possi-
ble 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 (SABATH et al. 1996).

The time course of trans-resveratrol and e-viniferin
accumulation in berries was similar, with the maximum
amounts 4 d after inoculation. The late e-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 e-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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