Stilbene Production by *Vitis vinifera* Cell Suspension Cultures: Methyl Jasmonate Induction and ^{13}C Biolabeling

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Vitis vinifera cells grown in vitro respond to methyl jasmonate (MeJa) with enhancement of phytoalexin accumulation. MeJa added during the first half of the exponential growth phase increased the intracellular accumulation of stilbenes, mainly (*Z*)- and (*E*)-piceid (3,5,4′-trihydroxystilbene-3-*O*-*β*-glucoside), in strains grown in either maintenance medium or polyphenol inductive medium. Plant cell cultures were used to produce isotopically (^{13}C) labeled stilbenes by addition of ^{13}C phenylalanine at different time intervals and in various concentrations. Incorporation of ^{13}C phenylalanine into stilbenes was measured by EA-IRMS, and three successive 2-mM phenylalanine additions provided the best result (66%). This allowed production of ^{13}C labeled *Vitis vinifera* phenolic compounds for investigation of their absorption in humans.

Vitis vinifera cell suspension cultures accumulate anthocyanins, proanthocyanidins, catechins, and stilbenes.[1,2] The principal stilbenes characterized in these cultures, (*E*)- and (*Z*)-resveratrol (3,5,4′-trihydroxystilbene) and their glycosides, are present in grape berries and red wine. These are probably the most important foodstuffs containing these substances.[3,4] Stilbenes have attracted a great deal of interest, for they may exert a protective effect against atherogenesis through their potent antioxidant properties on human low-density lipoproteins.[5] Moreover, (*E*)-resveratrol has potential cancer chemopreventive activity.[6] We therefore undertook the production of polyphenols by grape cells to obtain isotopically labeled compounds indispensable for investigations into their bioavailability and pharmacokinetics.

Jasmonic acid and its methyl ester (MeJa) have been proposed as key compounds of the signal transduction system involved in the formation of high and low molecular weight compounds of plant defense reactions.[7,8] Plant cell suspension cultures can be elicited by jasmonates resulting in increased accumulation of secondary metabolites.[7,9,10]

In the present work, we supplied *Vitis vinifera* cell cultures with MeJa in order to demonstrate the induction of stilbene phytoalexins and to optimize the in vitro production of these phenolic compounds. We further investigated the incorporation of ^{13}C-phenylalanine into stilbenes by grape cells using carbon isotopic measurement with EA-IRMS.

The response of grape cell cultures to MeJa treatment was first studied. MeJa was added to various *Vitis vinifera* cell suspensions: the GT strain that biosynthesizes high levels of anthocyanins [11] and two white strains (CS4, CS6). To determine the optimal conditions for stilbene production, we investigated the final concentration of MeJa (10−150 μM) and the time of addition (days 5, 6, 8, and 10) with grape cells grown in both maintenance and induction media. We present the production of (*Z*)- and (*E*)-piceid, which together accounted for around 90−95% of the total stilbenes accumulated in the three strains (Table 1).

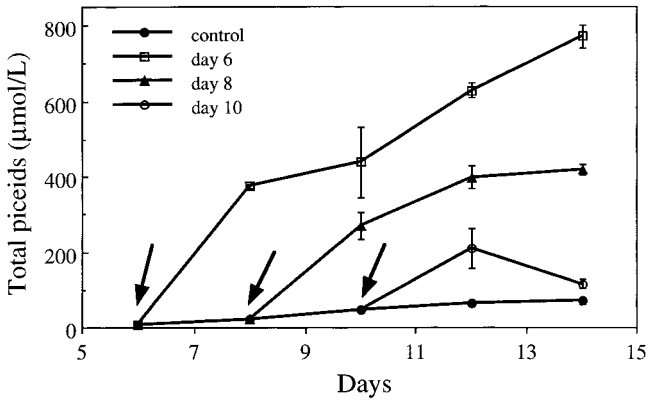

Figure 1. Effect of addition of MeJa (25 μM) on the kinetics of total piceid production in grape cell suspension culture (CS4) in IM1. The arrows show the time period for the addition of MeJa (at day 6, 8 or 10). Results are the means ± standard deviation of 3 replicates.

The results in the maintenance medium clearly indicate that the accumulation of piceids was stimulated by MeJa. However, the amount obtained was not as large as found in cells grown in an induction medium (IM1) in strains GT and CS4. The above experiment was repeated using IM1, where MeJa was added from the beginning to the middle of the exponential growth phase. Stilbene accumulation was notably induced in grape cells: the optimal concentration in all strains was 25 μM. The induction of piceid production was maximal when MeJa was added from the beginning (at day 6) to the middle (at day 8) of the exponential growth phase. In CS4 strain, total piceid production was higher than in GT and CS6 strains. This significantly increased 11-fold when the elicitor was added at day 6 and the cells harvested at day 14, and reached 1% of the dry weight (Figure 1).

MeJa also led to an increase in the accumulation of (*E*)-resveratroloside (resveratrol-4′-*O*-*β*-D-glucopyranoside),[12] whereas the low levels of the (*Z*) structure of the latter, (*E*)- and (*Z*)-astringin (3,5,3′,4′-tetrahydroxystilbene-3-*O*-*β*-glucoside), and (*E*)- and (*Z*)-resveratrol were not affected.

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Table 1. Effect of MeJa Addition on Cell Growth and Piceid Production in GT, CS4, and CS6 Strains of *Vitis vinifera*[a]

	strain								
	GT			CS4			CS6		
medium	total piceids (μmol/L)	dry wt (g/L)	optimal MeJa concentration (μM)	total piceids (μmol/L)	dry wt (g/L)	optimal MeJa concentration (μM)	total piceids (μmol/L)	dry wt (g/L)	optimal MeJa concentration (μM)
maintenance medium									
control	59 ± 4	10.8		35 ± 2	9.2		27 ± 3	10.6	
MeJa	142 ± 8	9.1	50	41 ± 3	9.6	10	63 ± 1	10.2	10
induction medium									
control	162 ± 11	23.4		70 ± 8	30.8		9 ± 3	22.1	
MeJa	495 ± 36	18.0	25	771 ± 31	27.8	25	237 ± 16	20.0	25

[a] Results are the means of three replicates (± S.D. for piceid levels) and are only presented in optimal conditions. MeJa was added at day 5, and the cells were harvested at day 7 for the three strains in mainenance medium. This addition in the induction medium was done at day 6, and the cells were harvested at day 12 (GT and CS6 strains) or 14 (CS4 strain).

Table 2. Production and Percentage of ^{13}C Enrichment of Piceids after Incorporation of [1-^{13}C] L-Phenylalanine (1−^{13}C PHE) by *Vitis vinifera* Cells (CS4 strain) Grown in IM1[a]

1−^{13}C PHE (mM)	day of addition	total piceids (μmol/L)	(*E*)-piceid ^{13}C enrichment (%)
1	6	881 ± 6	32 ± 0.9
1, 1	6, 8	902 ± 6	36 ± 0.3
1, 1, 1	6, 8, 10	814 ± 5	47 ± 0.3
2	6	934 ± 19	40 ± 0.3
2, 1	6, 8	730 ± 21	48 ± 1.2
2, 1, 1	6, 8, 10	907 ± 44	54 ± 3.9
2, 2	6, 8	905 ± 19	58 ± 0.6
2, 2, 1	6, 8, 10	1020 ± 33	58 ± 3.4
2, 2, 2	6, 8, 10	1011 ± 18	66 ± 3.5

[a] Data are the means of four replicates ± S. D.

The amounts of all stilbenes in the culture medium were negligible in both MeJa and control cultures.

Then we investigated the production of ^{13}C-labeled stilbenes using the optimal conditions for total piceid production, that is, when MeJa (25 μM) was applied to the CS4 strain (IM1) at day 6. Previous work has shown that addition of phenylalanine at the beginning of the exponential growth phase results in optimal incorporation into anthocyanins in *Vitis vinifera* cells.[13] Various concentrations of nonlabeled phenylalanine (1 to 10 mM, final concentration) were added at day 6 to the CS4 strain in the optimal conditions of piceid production. No modification in the grape cell growth was observed until addition of 6 mM of phenylalanine. However, a rapid decrease in total piceid content (80%) was recorded from 2 mM to 4 mM. To obtain optimal incorporation of [1-^{13}C]-L-phenylalanine into piceids, this precursor was added at 1 mM or 2 mM, at day 6, days 6 and 8, or days 6, 8, and 10. Piceids were purified by a combination of chromatographic techniques. Percentages of enrichment were determined by EA-IRMS and presented for (*E*)-piceid in Table 2. Indeed, similar results were found for both piceids. A higher percentage of ^{13}C enrichment of (*E*)-piceid was observed after a 2-mM addition at day 6 than with 1 mM at the same time. This was true in all cases (1, 2, or 3 additions). A second and third addition of 2 mM of labeled phenylalanine also increased the percentage up to 66%. Moreover, total piceid production increased 1.4-fold from control to three 2-mM phenylalanine additions. We have previously reported a stimulating effect of this precursor on the accumulation of anthocyanins in the GT strain.[13] The results obtained with incorporation of ^{13}C-labeled phenylalanine into stilbenes by this strain were similar to those obtained with the CS4 strain. The former will allow production of various labeled polyphenols, whereas the latter mainly produces stilbenes, which can easily be purified in the absence of anthocyanins.

Thus, MeJa is able to induce the biosynthesis of stilbenes and can be used to optimize the in vitro production of these compounds. This finding is consistent with the postulate that jasmonates are key components of intracellular signaling in response to pathogenic attacks in the grapevine. Indeed, elicitation by *Botrytis cinerea* can induce de novo synthesis of stilbene synthase in cultured grapevine cells [14] and the corresponding gene in transgenic tobacco. [15] We are now testing the potential for prophylactic MeJa or derivative treatments in grapevine to increase its resistance to fungal pathogens.

It is now possible to produce isotopically ^{13}C- or ^{14}C-labeled stilbenes for investigation of their absorption and in vivo metabolism in humans and animals. Both (*E*)- and (*Z*)-resveratrol are easily obtained by enzymatic hydrolysis of piceids.

Experimental Section

General Experimental Procedures. ^{13}C enrichments were measured by EA-IRMS using a Carlo Erba (C2500) elemental analyzer coupled to a Delta Plus mass spectrometer from Finnigan-mat.

Cell Culture. Three strains of *Vitis vinifera* (L.) were used: the GT strain, a cell suspension culture of Gamay Fréaux var. Teinturier established as described previously, [1] and two strains of Cabernet Sauvignon (CS4 and CS6) initiated in our laboratory from petioles of various plants (Chateau Cabannieux, Bordeaux) in 1994. These three strains were maintained under continuous fluorescent light (5000 lux) at 25 ± 1 °C in 250 mL Erlenmeyer flasks containing 50 mL of cell suspension on an orbital shaker (100 rpm). The maintenance medium (MM) contained macroelements, microelements, vitamins, 58 mM sucrose, and 250 mg/L casein hydrolysate.[1] It was supplemented with either 0.5 μM 1-naphthaleneacetic acid and 1 μM kinetin (MM1 for GT strain) or 2.5 μM 1-naphthaleneacetic acid and 0.5 μM 6-benzylaminopurine (MM2 for CS4 and CS6 strains). For experimental purposes, we inoculated a 7-day-old cell suspension into the induction medium (IM1) at a 1:8 (v/v) ratio.[1] This was similar to MM1, but contained 2 mM $(NH_4)_2SO_4$, 2.2 mM NaH_2PO_4, 2 mM $MgSO_4$, and 175 mM sucrose.

Elicitor Treatment and Precursor Feeding. MeJa and [1-^{13}C] L-phenylalanine were purchased from Sigma (France) and Eurisotop (CEA, France). They were dissolved in MeOH and DMSO−H_2O (15:85), respectively, and filter-sterilized. Control cultures received the corresponding vehicle solvent at a final concentration that did not exceed 0.2% (MeOH) or 0.5% (DMSO). These compounds were applied at different growth phases, and cells were harvested by vacuum filtration, rapidly washed with cold distilled H_2O, weighed, and stored at −20 °C until analysis.

Quantification of Stilbenes. Stilbenes were extracted from freeze-dried cells (50 mg) overnight with MeOH (4 mL) at +4 °C and from medium (10 mL) with ethyl acetate (10 mL). The following operations were performed as described earlier.[2] Extracts were chromatographed on a cation-exchange resin column to obtain a crude stilbene mixture. Analysis of stilbenes was performed by HPLC on a reversed-phase C_{18} column. The chromatogram was monitored at 286 and 306 nm. Stilbene contents were estimated from a calibration curve that was prepared with standards of (*E*)- and (*Z*)-resveratrol, piceid, astringin, and resveratroloside, purified from cultured grape cells.

Purification of Stilbenes and Measurement of ^{13}C Enrichment. Freeze-dried cells were extracted (× 2) with MeOH. The following operations were performed as described earlier.[12] Extracts were chromatographed on a Sephadex LH-20 resin column to obtain two main fractions: (*E*)-stilbenes and (*Z*)-stilbenes. Then (*E*)- and (*Z*)-piceid were purified by semi-preparative HPLC on a reversed-phase C_{18} column. The chromatogram was monitored at 286 and 306 nm. The structures of piceids and the C-1 labeling were verified by ^{13}C NMR, and the percentage of ^{13}C enrichment was measured by EA-IRMS. Samples were injected via an automatic injector into the elemental analyzer and totally converted to CO_2. The CO_2 was transferred to the IRMS using helium (120 mL/min). The different isotopomers were separated by a uniform magnetic field and collected in three different collectors at *m*/*z* 44, 45, and 46. Known percentage enrichment of [1−^{13}C] L-phenylalanine (99%) was regularly analyzed to verify the validity of the method. ^{13}C atom percentage was tranformed to ^{13}C enrichment percentage (% of [1−^{13}C] piceid) with the formula

$$^{13}C \text{ enrichment } \% = {}^{13}C \text{ atom } \% \times 20 \text{ (number of C per molecule)}$$

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References and Notes

(1) Decendit, A.; Mérillon, J.-M. *Plant Cell Rep*. **1996**, *15*, 762−765.
(2) Waffo Teguo, P.; Decendit, A.; Krisa, S.; Deffieux, G.; Vercauteren, J.; Mérillon, J.-M. *J. Nat. Prod*. **1996**, *59*, 1189−1191.
(3) Mattivi, F.; Reniero, F.; Korhammer, S. *J. Agric. Food Chem*. **1995**, *43*, 1820−1823.
(4) Goldberg, D. M.; Karumanchiri, E. Ng. A.; Diamandis, E. P.; Soleas, G. *Am. J. Enol. Vitic.* **1996**, *68*, 1688−1694.
(5) Fauconneau, B.; Waffo Téguo, P.; Huguet, F.; Barrier, L.; Decendit, A.; Mérillon, J.-M. *Life Sci.* **1997**, *61*, 2103−2110.
(6) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218−220.
(7) Gundlach, H.; Muller, J. M.; Kutchan, T. M.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2389−2393.
(8) Creelman, R. A.; Mullet, J. E. *Plant Cell* **1997**, *9*, 1211−1223.
(9) Ellard-Ivey, M.; Douglas, C. J. *Plant Physiol*. **1996**, *112*, 183−192.
(10) Yazaki, K.; Takeda, K.; Tabata, M. *Plant Cell Physiol*. **1997**, *38*, 776−782.
(11) Larronde, F.; Krisa, S.; Decendit, A.; Chèze, C.; Deffieux, G.; Mérillon, J.-M. *Plant Cell Rep*. **1998**, *17*, 946−950.
(12) Waffo Teguo, P.; Fauconneau, B.; Deffieux, G.; Huguet, F.; Vercauteren, J.; Mérillon. J.-M. *J. Nat. Prod*. **1998**, *61*, 655−657.
(13) Krisa, S.; Waffo Teguo, P.; Decendit, A.; Deffieux, G.; Vercauteren, J.; Mérillon, J.-M. *Phytochemistry*. **1999**, *55*, 651−656.
(14) Liswidowati, F.; Melchior, F.; Hohmann, F.; Schwer, B.; Kindl, H. *Planta* **1991**, *183*, 307−314.
(15) Hain, R.; Reif, H. J.; Krause, E.; Langebartels, R.; Kindl, H.; Vornam, B.; Wiese, W.; Schmelzer, E.; Schreier, P. H.; Stöcker, R. H.; Stenzel, K. *Nature* **1993**, *361*, 153−156.

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