Control of anthocyanin biosynthesis pathway gene expression by eutypine, a toxin from *Eutypa lata*, in grape cell tissue cultures

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Summary

Eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde, is a toxin produced by *Eutypa lata*, the causal agent of Eutypa dieback in grapevine. The effect of the toxin on anthocyanin synthesis has been investigated in *Vitis vinifera* cv. Gamay cell cultures. At concentrations higher than 200 μmol/L, eutypine reduced anthocyanin accumulation in cells. The reduction in anthocyanin accumulation was proportional to the eutypine concentrations and HPLC analysis showed that eutypine affected the levels of all anthocyanins. The effect of eutypine application on the expression of five genes of the anthocyanin biosynthesis pathway, including chalcone synthase (*CHS*), flavonone-3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin dioxygenase (*LDOX*), and UDP glucose-flavonoid 3-*O*-glucosyl transferase (*UFGT*) was determined. Expression of *CHS, F3H, DFR* and *LDOX* was not affected by the addition of eutypine to grapevine cell cultures. In contrast, expression of the *UFGT* gene was dramatically inhibited by the toxin. These results suggest that in grapevine cell cultures, eutypine strongly affects anthocyanin accumulation by inhibiting *UFGT* gene expression. The mechanism of action of eutypine is discussed.

Key words: Anthocyanins – Eutypa dieback – *Eutypa lata* – eutypine – grapevine – toxin – UDP glucose-flavonoid 3-*O*-glucosyl transferase (*UFGT*)

Abbreviations: *CHS* = chalcone synthase. – *DFR* = dihydroflavonol 4-reductase. – *F3H* = flavonone-3-hydroxylase. – HPLC = high pressure liquid chromatography. – *LDOX* = leucoanthocyanidin dioxygenase. – *UFGT* = UDP glucose-flavonoid 3-*O*-glucosyl transferase

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Introduction

Eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzalde-hyde, is a toxin produced by the ascomycete fungus *Eutypa lata* (Pers. : Fr.) Tul., the causal agent of Eutypa dieback (Tey-Rulh et al. 1991). It has been demonstrated that the toxin plays an important role in the development of disease symptoms, which affects the growth of the branches and the development of the grape berries (Deswarte et al. 1996, Fallot et al. 1997). Studies in a number of vineyards throughout the world have shown that Eutypa dieback reduces the yield of grapevines and affects the quality of the grape berries (Moller and Kasimatis 1981).

The colour of red and black grapes results from the accumulation of anthocyanins that are usually located in the skin of the berry. It has been demonstrated that the quantity and the quality of anthocyanins in grape berries greatly affect the quality of red wines. Grapevines usually produce 3-monoglu-coside, 3-acetylglucoside and 3-p-coumarylglucoside derivatives of the aglycones delphinidin, cyanidin, peonidin, petunidin and malvidin (Mazza and Miniati 1993). The pathway leading to the production of anthocyanins in grapevine has been described and cDNAs for genes from the flavonoid pathway including chalcone synthase (*CHS*), flavonone-3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin dioxygenase (*LDOX*) and UDP glucose-flavonoid 3-*O*-glucosyl transferase (*UFGT*) have been isolated from grapes (Sparvoli et al. 1994). Expression of these genes has been detected in a number of grapevine tissues (Boss et al. 1996a).

The aim of this study was to investigate the effects of eutypine on anthocyanin production. Callus tissue cultures of *Vitis vinifera* cv. Gamay were used to study the effect of eutypine on the anthocyanin profiles and to analyse the effect of eutypine on the expression of *CHS, F3H, DFR, LDOX* and *UFGT* genes from the anthocyanin biosynthetic pathway. We report that eutypine affected the anthocyanin biosynthetic pathway in cell cultures by inhibiting *UFGT* gene expression. These data open new perspectives for the understanding of the toxic action of eutypine.

Material and Methods

Grapevine cell cultures

Callus tissue cultures of *Vitis vinifera* cv. Gamay, originating from the skin of grape berries, were grown in solidified medium (0.7 % agar) as previously described by Ambid et al. (1983). The grapevine cultures were maintained at 25 °C, under a 16-h light / 8-h dark cycle with a photon flux of 100 μmol m^{-2} s^{-1} (Osram L36 W/36 Nature tubes). Tissue cultures that were 20 days old were used in all experiments and cultured in a fresh medium containing various concentrations of eutypine.

Anthocyanin extraction and HPLC analysis

The tissue cultures of *Vitis vinifera* cv. Gamay were ground in liquid nitrogen using a mortar and pestle. Anthocyanins were extracted with methanol at −20 °C, for 1 h. The tissues were pelleted by centrifugation at 10,000 *g* for 15 min at 4 °C and the supernatant was retained for further HPLC analysis. The HPLC method used for separation of individual anthocyanins was close to that described by Boss et al. (1996 b) with some modifications. Chromatographic analysis of anthocyanin compounds used a Waters HPLC system consisting of a Waters 600 solvent delivery system, a Waters 717 plus autosampler and a Waters 2487 Dual λ absorbance detector. Data were acquired and processed in a Waters Millenium^{32} workstation, which gave retention times and measured peak areas. A Prontosil Eurobond C *18* column (250×4 mm, 5 µm) was used for separation. The column temperature was thermostated at 35 °C. A linear gradient as shown in Table 1 was used for elution. The mobile phase was a mixture of water, 1.4 % perchloric acid and methanol. The flow rate was 1.5 mL/min during the run time. Before the injection, samples were filtered through a 0.22 µm nylon filter (Millipore). Injection of 15 µL of each sample was made automatically by the autosampler, and the needle was cleaned with water/methanol (50/50 %). Total anthocyanins were determined by measuring absorbance at 520 nm. The anthocyanin concentrations were calculated using the extinction coefficient of malvidin 3-glucoside as reference [ε = 38,000 L M^{-1} cm^{-1} (Hrazdina et al. 1984)]. All measurements were made in triplicate.

RNA extraction and Northern blot

Total RNA was extracted from callus tissues according to the method described by Boss et al. (1996 b). Aliquots of 15 µg RNA were separated in 1.2 % agarose gel, transferred onto GeneScreen Plus membranes according the manufacturer's procedure (Dupont de Nemours, Switzerland), and cross-linked with a UV crosslinker (Amersham, UK). Pre-hybridisations were performed for 4 h, at 42 °C, in 5 × SSPE, 50 % (w/v) deionised formamide, 1 % SDS, 10 % Dextran sulphate-Na salt (MW 500.000) and 10 µg/mL denatured salmon sperm. cDNA of grape anthocyanin pathway genes, cloned by Sparvoli et al. (1994), were labelled using a random prime labelling kit (Ready-To-Go DNA labelling Beads, -dCTP, Amersham, Les Ulis, France) and used as a probe. After hybridisation for 15 h at 42 °C, the membranes were washed at 42 °C, twice in 2 × SSC, 0.1 % SDS for 10 min, and then for 15 min in 1×SSC and 0.1 % SDS at 42 °C and exposed to X-ray film (Hyper film, Amersham Pharmacia Biotech) using

Table 1. Linear gradient used for anthocyanin compound separation by HPLC. Solvents A, B and C: ‹A› was water; ‹B› was perchloric acid 1,4 % and ‹C› was methanol.

| Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	45	30	25
3	35	30	35
15	15	30	55
26	15	30	55
30	45	30	25
35	45	30	25
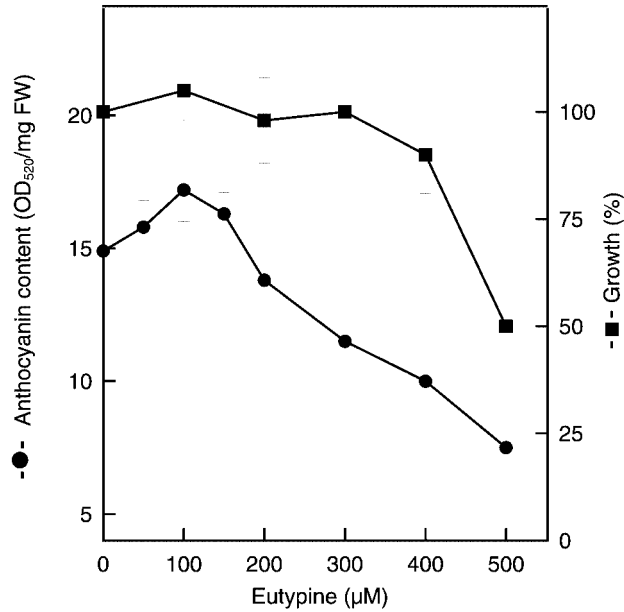
Figure 1. Effects of eutypine on growth and anthocyanin concentrations in grapevine tissues. Anthocyanin contents were measured after 7 days of culture in the presence of eutypine. Data are the means ± SE of results from three independent experiments.

intensifying screens at $-80\,^{\circ}C$. The membranes were also hybridised with an 18 S-RNA probe.

Results and Discussion

The grapevine cells were cultured in the presence of various concentrations of eutypine ranging from 0 to 500 µmol/L and anthocyanin contents were analysed after 7 days of culture. The presence of eutypine at concentrations less than 200 µmol/L did not significantly affect the anthocyanin levels in grapevine cell cultures after 7 days of culture (Fig. 1). At concentrations higher than 200 µmol/L, eutypine induced a substantial decrease in anthocyanin accumulation, and the reduction of anthocyanin content was proportional to the eutypine concentration. In the presence of 400 µmol/L eutypine, the anthocyanin concentration was reduced by 30 %, whereas the growth of tissue cultures was not affected by the presence of the toxin. This eutypine concentration was used for the further experiments.

HPLC analysis showed that sixteen anthocyanins were present in the samples and fifteen of these were identified by comparing their retention times and elution order with previous data of grape and wine anthocyanins (Larry et al. 1978,
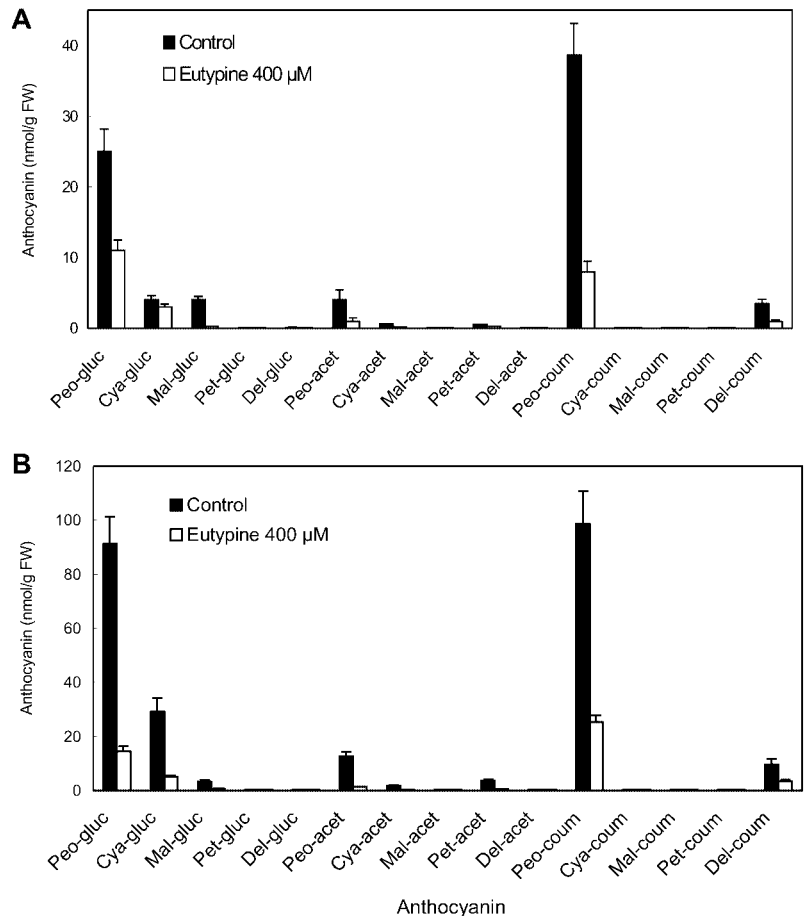
Figure 2. Anthocyanin contents of grapevine tissues cultured in the presence of 400 µmol/L eutypine for 3 (A) and 7 (B) days. The anthocyanin were assayed by HPLC. Data are means ± SE of results from three independent experiments.
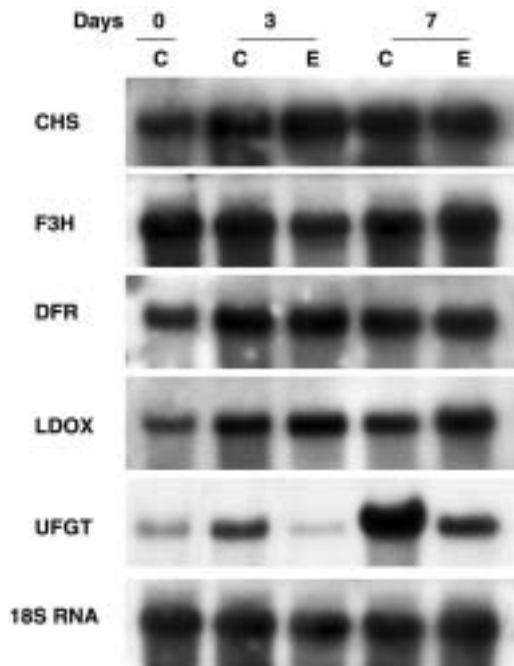
Figure 3. Northern blots of five anthocyanin biosynthesis genes in grapevine tissues cultured in the absence (C) or presence (E) of 400 µmol/L eutypine for 3 and 7 days. CHS: chalcone synthase, F3H: flavanone-3-hydroxylase, DFR: dihydroflavonol-4-reductase, LDOX: leucoanthocyanidin dioxygenase, UFGT: UDP-glucose:3-*O*-flavonoid glucosyl transferase.

Boss et al. 1996 b, Luczkiewicz and Cisowski 1998). The most abundant anthocyanins present in the callus tissues were peonidin derivatives and cyanidin-3-glucoside (Fig. 2). For the peonidin anthocyanins, 3-monoglucoside and 3-p-coumarylglucoside derivatives were the major contributors. They represented 62 % and 70 % of the total anthocyanins in 3- and 7-day-old grapevine cultures, respectively. Malvidin-3-glucoside and other malvidin derivatives were found to be present at low concentrations. Furthermore, during cell culture, the anthocyanin contents increased, but the proportions for the different anthocyanins were not significantly affected (Fig. 2). The anthocyanin profile present in our cell cultures was similar to that described in other *V. vinifera* cell cultures (Taylor and Briggs 1990, Kao et al. 1996). In the presence of 400 µmol/L eutypine, the concentration of each anthocyanin was strongly reduced, the inhibition affecting all anthocyanins (Fig. 2). Peonidin-3-monoglucoside was reduced by 52 % and 76 % after 3 and 7 days of culture, respectively. Similarly, eutypine caused a 74 % reduction of the peonidine-3-p-coumarylglucoside concentration after 7 days of culture. Nevertheless, the proportion of each anthocyanin in the total anthocyanins was not significantly affected by the presence of eutypine.

The effect of eutypine on the expression of five genes (*CHS, F3H, DFR, LDOX* and *UFGT*) involved in anthocyanin biosynthesis was investigated in grapevine cell cultures after 3 and 7 days of culture. In controls, Northern blot analysis showed that all of the anthocyanin pathway genes examined, except the *UFGT* gene, were expressed at the same level at days 3 and 7 in the grapevine cultures. In contrast, *UFGT* gene expression increased during development, an increase that coincided with the accumulation of anthocyanin in the tissues. The presence of 400 µmol/L eutypine failed to affect the expression of *CHS, F3H, DFR* and *LDOX* genes. The level of expression of these genes appeared similar to that of the control after 3 or 7 days of culture. In contrast, in the presence of eutypine, the expression of the *UFGT* gene was dramatically inhibited after 3 and 7 days of culture. The inhibition of *UFGT* gene expression by eutypine appears to coincide with the decrease in anthocyanin accumulation. It has been suggested that the *UFGT* gene plays a crucial role in the control of anthocyanin biosynthesis in grapevine (Boss et al. 1996 a). Our results support this hypothesis. On the other hand, Amborabe et al. (2001) reported that eutypine does not modify phenylalanine ammonia lyase activity, one of the first enzymes involved in the production of phenylpropanoid compounds leading to anthocyanin biosynthesis. Thus, it seems that eutypine has no effect on the first step of anthocyanin biosynthesis. The mechanism of action of eutypine on the expression of the *UFGT* gene is not known. However, the toxin could inhibit its expression directly or indirectly by affecting the expression of regulatory genes that control the expression of structural genes including the *UFGT* gene of the anthocyanin biosynthetic pathway. Indeed, in grapevine berries, a coordinated induction of the structural genes of the anthocyanin biosynthetic pathway has been suggested (Boss et al. 1996 b).

In conclusion, the results of this work show that eutypine affects the accumulation of anthocyanins in the grapevine cell cultures by inhibiting the expression of the *UFGT* gene. The inhibition is strongly dependent on the toxin concentration. These results clearly show that eutypine can inhibit anthocyanin production in grapevine and that in diseased plants, eutypine produced by the parasitic fungus present in the trunk and transported by the sap to the herbaceous part may be involved in decrease of grape berry quality.

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