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Metabolisation of eutypine by plant tissues: an HPLC determination

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Abstract

Eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde, is a toxin produced by *Eutypa lata*, the causal agent of eutypa dieback of grapevine. The tolerance of some grapevine cultivars to the disease has been ascribed to the potential reduction of eutypine into its corresponding non-toxic alcohol, eutypinol. In the present study, eutypine biotransformation in different tissues of grapevine was investigated by HPLC and LC-MS. Grape callus tissues were able to biotransform eutypine into eutypinol within the first 3 h of culture. The grape plantlets cultured in vitro can also transform eutypine into eutypinol. Grape plantlet leaves do not have any effect on the uptake of eutypine, which goes through the tissues following a concentration gradient. Results revealed that the toxicity of eutypine in grape tissues is an active process showing that eutypinol is rapidly metabolised into other compounds. The use of micro-cuttings and in vitro plants showed that eutypine strongly accumulates in the bottom part of the diseased plant stems.

Keywords Eutypine; Eutypinol; Eutypa dieback; *Eutypa lata*; Grapevine; HPLC

1. Introduction

The ascomycete fungus *Eutypa lata*, responsible for eutypa dieback, is having important economic consequences on grapevines throughout the world [1,2]. The disease is characterised by dwarfed or withered new growth, marginal necrosis of the leaves, dryness of the inflorescences, saratorial necrosis of the stems, burnishing dryness and death of one or more branches [3]. The fungus synthesises a toxin, named eutypine, structurally characterised as 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde [4,5] (Fig. 1). The toxin is transported by the sap to the herbaceous parts of the vine and plays a prominent role in the expression of eutypa dieback symptoms [3]. Previous works brought some information on the mode of action of eutypine at the cellular level and it has been shown that

eutypine exhibits a protonophoric activity [6,7]. Eutypine levels in diseased plant extracts might explain differences in expression of symptoms according to different culture or climatic parameters. The internal toxic concentration of eutypine, which can cause toxic symptoms in plant tissues, is not known yet.

It was established that in the grapevine cells, eutypine is metabolised into another compound identified as 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzyl alcohol, or eutypinol (Fig. 1). This compound does not exhibit toxic effects towards the grapevine and has no protonophoric activity. Reduction of the carbonyl group of eutypine into an alcohol function is catalysed by eutypine reductase. This enzyme was partially purified from grapevine cell suspensions [8].

Our aim was to characterise the biotransformation of eutypine into eutypinol in grape plant tissues. The time-course of eutypine biotransformation into eutypinol was studied using different grape plant materials (callus cells, micro-cuttings, plantlets) and culture media. Sample analyses were carried out with HPLC and LC-MS.

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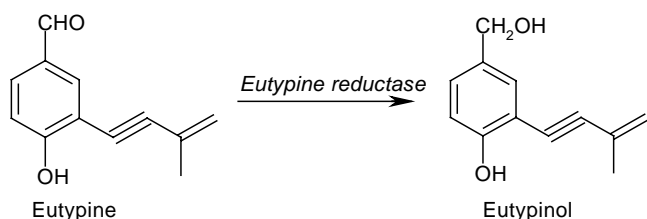


Fig. 1. The biotransformation of eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde, into its corresponding alcohol metabolite eutypinol, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzyl alcohol.

2. Experimental

2.1. Chemicals

Eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde, was synthesised by Raffaele Tabacchi (Neuchâtel, Switzerland), as previously described [9].

2.2. *In vitro* culture incubation conditions

In all the experiments, *in vitro* cultures were incubated at 25 °C, under a 16 h light/8 h dark cycle with a photon flux of 100 $\mu\text{M m}^{-2} \text{s}^{-1}$ (Osram L36 W 36 Nature tubes) and 70% humidity.

2.3. Plant material preparation

2.3.1. Grape callus cell cultures

Callus cell cultures of *Vitis vinifera* L. cv. Gamay, originating from grape berry thin skin, was grown in solidified medium (0.7% agar) as previously described [10]. After 21 days (exponential growth phase) of culture, calli were transferred to fresh medium containing various concentrations of eutypine.

2.3.2. Grape micro-cuttings

Vitro plants of *V. vinifera* cv. Ugni-blanc were cultured as previously described [11]. After 5 weeks of culture, leaves and roots of each plantlet were removed under sterile conditions. Each main stem was divided into micro-cuttings each of which carried one bud. The micro-cuttings were cultured in the presence of 500 μM eutypine in petridishes containing solidified medium (0.7% agar) as previously described [10].

2.3.3. Grape plantlets

Vitro plants of *V. vinifera* cv. Ugni-blanc were collected after 5 weeks of culture. Roots of each plantlet were removed under sterilised conditions to give a plantlet with three vegetal levels. Plantlets without roots were cultured in Magenta vessels containing 40 ml of Murashige and Skoog's medium culture as previously described [12] in the presence of eutypine at 500 μM . After 2, 5 and 10 days of culture, plantlets and culture media were collected. Each plantlet

contained three vegetal levels and in each level two leaves. Plantlets were divided into three parts starting with the basal level.

2.4. Eutypine extraction from plant materials

Plant materials in each experiment were ground using liquid nitrogen and were extracted with acetone (20 ml for 3.5 g fresh weight (FW) tissues) for 3 h under agitation. After filtrating, the residues were treated with dioxane (2.5 ml for 18 ml acetone) for 1 h under stirring. Pigments were eliminated by extraction with water (4 ml for 20 ml extract). Samples were placed at -18°C overnight and were then filtered using ash-free filter paper. Residual filtrate was evaporated to form a syrup-like residue which was diluted in 200 ml water and the pH adjusted to 5 with 0.1 M NaOH. Eutypine extraction was carried out with three portions of 100 ml ether. The raw etheric extracts were combined, dried by adding magnesium sulphate, filtered and then dried under vacuum at 35 °C using a rotary evaporator. The residue was suspended in 2 ml absolute ethanol for further analysis [13].

2.5. Eutypine extraction from culture media

The culture media were ultra-centrifuged at 100 000 g for 8 h at 10 °C. The liquid phase was harvested, adjusted to pH 5 using 0.1 M NaOH and extracted four times with 50 ml of ether. Organic phases were collected, dried by adding magnesium sulphate and filtered. The resulting filtrate was finally evaporated to dryness in a rotary evaporator under vacuum at 35 °C. After evaporating the solvent from the extract, the residue was dissolved in 2 ml absolute ethanol for further analysis.

2.6. HPLC analysis of the extract samples

Eutypine and its metabolites contained in the extracts were assayed by HPLC. HPLC conditions are quite close to those described previously [5] with some modifications. A Waters HPLC system was used 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 Millennium³² workstation, which gave retention times and measured peak areas. A Lichrosorb RP-C₁₈ column (5 μM , 150 mm \times 4.6 mm) was used to separate the eutypine and its metabolites. The column temperature was kept at 35 °C. The mobile phase was a mixture of solvents A and B: water and acetonitrile, respectively. A linear elution gradient was used as shown in Table 1. The flow rate was 1.5 ml min⁻¹ during the run time. Before the injection, samples were filtered through a 0.22 μM nylon filter (Millipore). The 15 μl injection volume of each sample was taken automatically by the autosampler. The needle was washed with water/methanol (50/50). The analysis was monitored at 260 nm.

Table 1
Linear gradient used for the separation of eutypine and its metabolites by HPLC

Time (min)	Water (%)	Acetonitrile (%)
0	70	30
10	65	35
20	60	40
30	70	30
35	0	100
40	0	100
45	30	70
60	70	30
65	70	30

2.7. Calibration curve of eutypine

Stock solution of 500 mM eutypine was prepared by dissolving 93 mg of pure eutypine in 1 ml of absolute ethanol. A concentration range of 0.4, 1, 6, 12, 24, 48, 145 and 241 μM eutypine solutions was prepared from the stock solution. Fifteen microliters of each dilution was injected three times to obtain the corresponding peak areas. A calibration curve fitted by plotting the mean of the three peak areas (y) against the related concentration of the standard solutions (x) gave the following regression equation: $y = 145\,225x$ with determination coefficient $r^2 = 0.9993$. This regression equation was used to quantify eutypine in samples.

2.8. LC-MS analysis of the extract samples

Eutypine and its metabolites present in the extracts were separated and determined by LC-MS (Service Central d'Analyse du CNRS, Vernaison, France). The analytical system consisted of a Hewlett-Packard/series HP 1100-MSD Model B. Data processed in a HP Chemstation (A.08.01-682). A Nucleodur Gravity C₁₈ (125 mm \times 3 mm; 3 μM) column from (Macherey-Nagel) at 50 °C was used for separation with a linear gradient for elution (Table 2). The mobile phase was a mixture of water and acetonitrile with a flow rate of 0.4 ml min⁻¹. Eutypine and its metabolites were detected at 262 nm. Electrospray mass detection was performed in the negative ion mode (ES⁻) (mass range: 100–1000 mass units) using MeOH + NH₄OH as eluent (0.2 ml min⁻¹). Pure eutypine and eutypinol were used as standards. LC retention times and mass spectra in the SIM mode allowed the identification of eutypine ($R_t = 9.71$ min, $[M - H]^- = 185$) and eutypinol ($R_t = 5.71$ min, $[M - H]^- = 187$) in extract samples.

Table 2
Linear gradient used for the LC-MS analysis of eutypine and its metabolites

Time (min)	Water (%)	Acetonitrile (%)
0	70	30
30	0	100

2.9. Statistical analysis

All samples were assayed in triplicate. Variance analysis (ANOVA) was performed using Jandel SigmaStat[®] Statale Software to express the experimental results. Means were compared with Duncan's multiple range test at $\alpha = 0.05$.

3. Results and discussion

3.1. Photo-stability of eutypine

The effect of light on eutypine stability was studied by incubating petridishes containing 25 ml of solidified medium (0.7% agar) using eutypine at 200 μM . The petridishes were placed under a light flux of 100 $\mu\text{M m}^{-2} \text{s}^{-1}$ or in darkness for 12 and 48 h. HPLC analysis revealed only the presence of eutypine and no significant difference between eutypine concentrations in the culture media incubated in the light or in the darkness after 12 and 48 h (Fig. 2). Statistical analysis showed no significant difference between the samples kept in light or darkness. Thus, in our *in vitro* culture incubation conditions light had no effect on eutypine stability.

3.2. Effect of agar concentration on eutypine uptake

The effect of the agar concentration on eutypine uptake by the tissues of *V. vinifera* cv. Gamay was studied by incubating callus on solidified medium using different agar concentrations (7, 9 and 12 g l⁻¹) in the presence of 200 μM eutypine. It was verified that the three agar concentrations did not affect callus growth. After 12 h of incubation, aliquots of culture medium were collected for eutypine extraction. HPLC analysis revealed the presence of eutypine and eutypinol in all the chromatograms of the medium extracts (Fig. 3). These data clearly show that the callus absorbed the eutypine from the culture media. The toxin is metabolised into eutypinol which diffuses from the callus

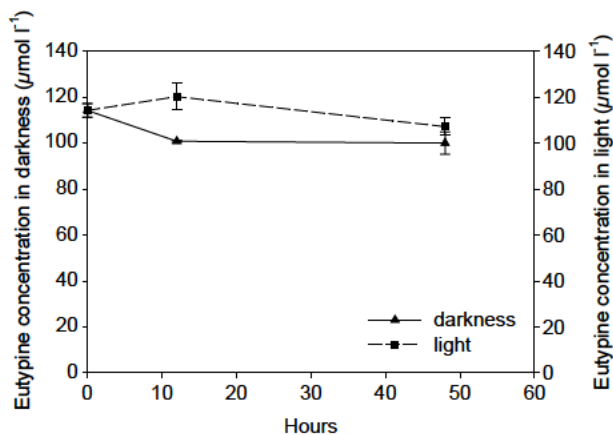


Fig. 2. Effect of light and darkness on the stability of eutypine in Gamay culture media in the presence of 200 μM eutypine (extracts from media).

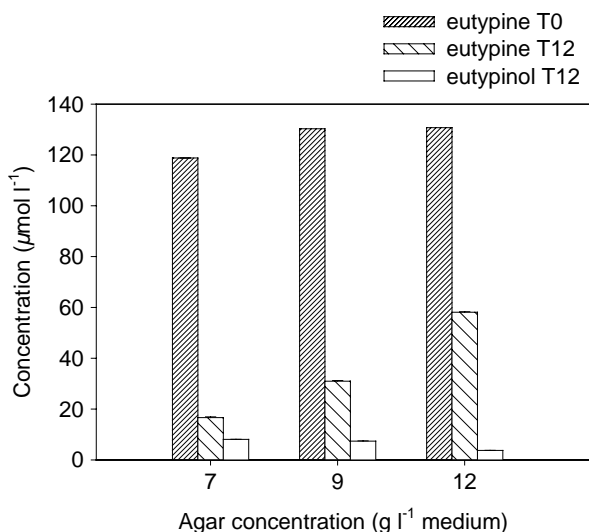


Fig. 3. Effect of agar concentration on eutypine absorption into the cells and eutypinol diffusion back to the culture media (extracts from media) (T0: at the beginning time, T12: after 12 h of culture).

tissue into the culture media (Fig. 3). These results also revealed a highly significant difference in eutypine uptake between the three agar concentrations studied. The lowest agar concentration (7 g l^{-1}) led to the highest eutypine absorption rate and of course the highest eutypinol diffusion from the callus to the media. The highest agar concentration (12 g l^{-1}) led to the lowest eutypine absorption by the callus, and hence the lowest eutypinol production. In the presence of 9 g l^{-1} agar, 75% of the eutypine in the culture media had been absorbed by the grapevine cells after 12 h of culture. This agar concentration (9 g l^{-1}) was selected to carry out the following experiments.

3.3. Eutypine biotransformation by grape callus tissues

To study the time-course of eutypine metabolisation, preliminary experiments using eutypine at a concentration of $200 \mu\text{M}$ were designed. Samples of callus tissues and culture media were collected after each hour of culture during 12-, 24- and 48-h cultures. The chromatogram of the callus extracts showed that Gamay callus tissues contained neither eutypine nor eutypinol during the experiment time. However, both were detected in the culture media as shown in Fig. 4. These data indicate that eutypine was absorbed and transformed rapidly by the callus. It was also observed that the eutypine concentration in the culture media decreased rapidly between 0 and 48 h of culture. The largest amount of eutypine was absorbed within the first period of culture between 0 and 12 h and the highest eutypinol concentration was detected after 48 h.

Because of the impossibility to detect either the eutypine or the eutypinol within the callus using a culture medium with $200 \mu\text{M}$ eutypine, two other experiments were carried out using a higher eutypine concentration ($500 \mu\text{M}$). Callus

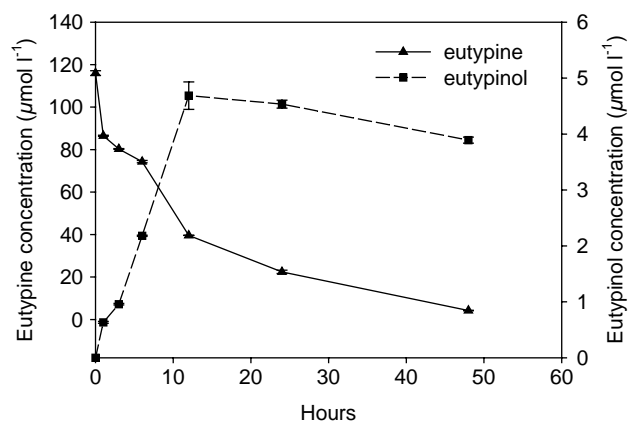


Fig. 4. Eutypine and eutypinol concentrations in Gamay culture media during the short experiment of Gamay callus culture in the presence of $200 \mu\text{M}$ eutypine.

tissues and culture media were collected in a first experiment after 1, 3 and 6 h of culture, extracted and analysed. HPLC analysis revealed the presence of both eutypine and eutypinol both in callus tissues and in the culture media 1 h after the beginning of the culture. The eutypine detection in the callus extract samples after 1 h of culture, indicated rapid absorption of eutypine by the callus tissues. Three hours later eutypinol increased to its highest level of $0.05 \mu\text{mol g}^{-1}$ fresh weight and then decreased after 6 h to $0.03 \mu\text{mol g}^{-1}$ FW within the callus tissues as shown in Fig. 5A. A small increase of eutypine concentration was observed in the callus tissues after 6 h of culture ($0.013 \mu\text{mol g}^{-1}$ FW). Moreover, results also show that within the callus tissues during the experiment period, the eutypinol concentration was higher than the eutypine concentration. In the same experiment, the eutypine concentration in culture media decreased rapidly between 0 and 1 h of culture. After that eutypine concentration remained stable for 6 h. The eutypinol concentration increased from 2.43 to $8.94 \mu\text{mol l}^{-1}$ after 1 and 6 h from the beginning of culture (Fig. 5B).

Eutypine and eutypinol in the different extracts were identified by LC-MS analysis. LC-MS scans displayed a molecular ion peak at $m/z = 185$ corresponding to eutypine and a molecular ion peak at $m/z = 187$ corresponding to eutypinol.

As shown in Fig. 4 and Fig. 5A and B, grapevine callus tissues are able to absorb eutypine and metabolise it into eutypinol very rapidly—within the first 3 h of culture. In this context, plant defence is known to be dependent on the efficiency of the rapid initiation and development of defence responses [14–16]. The effectiveness of defence response induction often depends more on its rapid initiation, development and accumulation than on the plant's ability to synthesise defence metabolites and proteins [14].

In order to study the stability and the behaviour of eutypine during long periods, a second experiment was carried out. Samples of callus and culture media were collected after 2, 5 and 10 days of culture. HPLC analysis revealed the

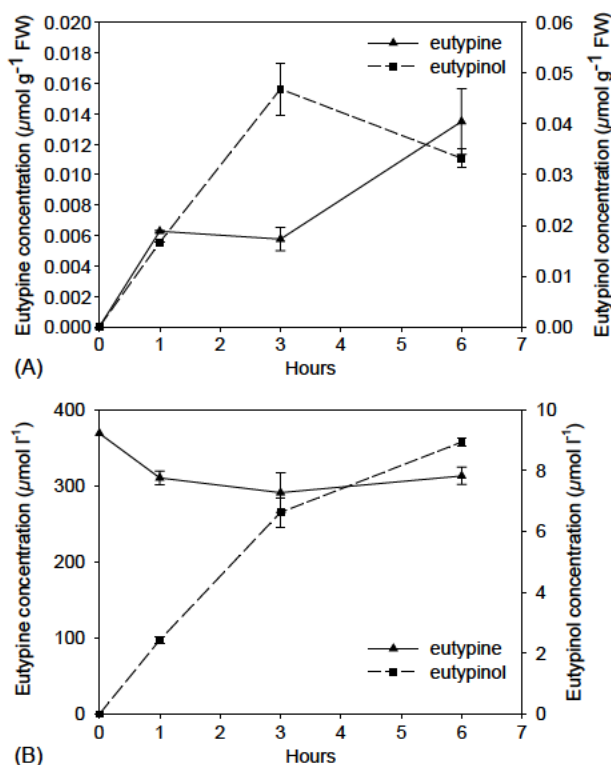


Fig. 5. (A) Eutypine and eutypinol concentration of Gamay callus tissues cultured in the presence of 500 μM eutypine after 1, 3 and 6 h. (B) Eutypine and eutypinol concentration in Gamay culture media during the short experiment using eutypine at 500 μM .

presence of eutypinol in the different callus extract samples in which eutypine was not detected (Fig. 6A). Both were observed by HPLC in the culture media (Fig. 6B). The highest eutypinol concentration within the callus tissues was detected after 48 h of culture with a decrease in the two following experimental days. The absence of eutypine from the callus tissues after 2 days and the presence of eutypinol suggest a rapid eutypine biotransformation into eutypinol. LC-MS scans of callus and culture media extracts confirmed the presence of eutypine $m/z = 185$ and eutypinol $m/z = 187$. It was not possible to detect eutypine by HPLC in the callus tissues but the high sensitivity of mass spectroscopy allowed its detection in callus extracts after 2 days of culture. This means that callus tissues after 2 days of culture contained very low amounts of eutypine.

3.4. Eutypine biotransformation by grape micro-cuttings

To study the capacity of grape tissues to metabolise eutypine, micro-cuttings of *V. vinifera* cv. Ugni-blanc were cultured on media containing 500 μM eutypine and samples were collected on days 2, 5 and 10. HPLC chromatograms showed that grape micro-cuttings generally contained a high concentration of eutypine over all experiment time (Fig. 7A). The highest concentration of eutypine was observed on day 2 in micro-cuttings 0.1 $\mu\text{mol g}^{-1}$ FW. Then

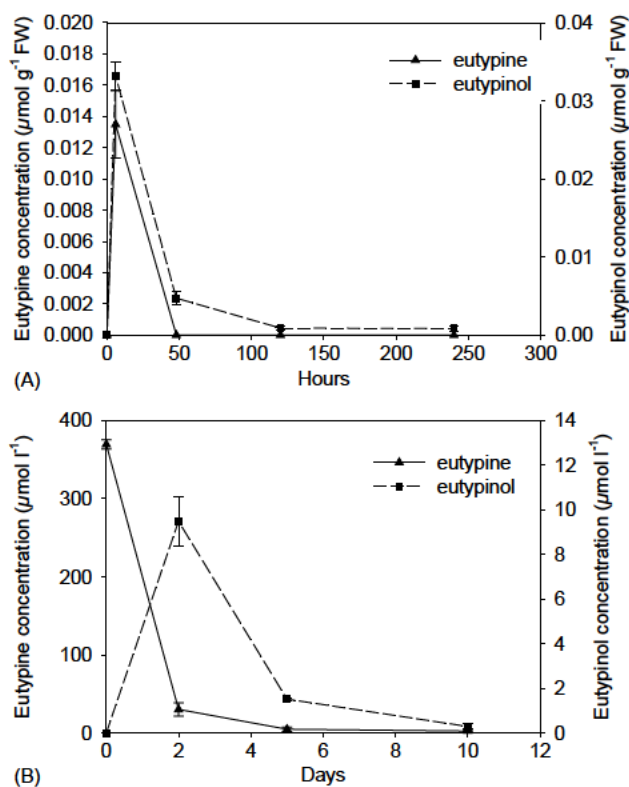


Fig. 6. (A) Time-course of eutypine and eutypinol concentrations in Gamay callus tissues cultured in the presence of 500 μM eutypine after 2, 5 and 10 days. (B) Eutypine and eutypinol concentrations in Gamay culture media during the long experiment.

eutypine concentration decreased to 0.05 and 0.04 $\mu\text{mol g}^{-1}$ FW in grape micro-cuttings after days 5 and 10, respectively. Eutypinol concentration reached a maximum value after 2 days of culture (0.01 $\mu\text{mol g}^{-1}$ FW) then decreased in the micro-cuttings to 0.003 and 0.001 $\mu\text{mol g}^{-1}$ FW after days 5 and 10, respectively (Fig. 7A). A strong decrease in eutypine concentration was observed within the first 2 days of culture in the grape micro-cuttings culture media (Fig. 7B). However, the highest eutypinol concentration was observed after 2 days, then eutypinol concentration decreased.

Comparative experiments on grapevine callus and micro-cutting showed the possibility to detect the eutypine within the micro-cutting tissues from 2 until 10 days of culture. However, it was impossible to detect the eutypine in the callus tissues, cultured with the same eutypine concentration, after 2 days of culture. Thus, eutypine absorption, metabolism and diffusion by grape micro-cutting are slower than that by callus tissues.

3.5. Eutypine uptake, transport and biotransformation by grape plantlets

This experiment was carried out to understand the transport and biotransformation of eutypine in *in vitro* plants of grapevine. Plantlets without roots were cultured on Murashige and Skoog medium containing 500 μM eutypine.

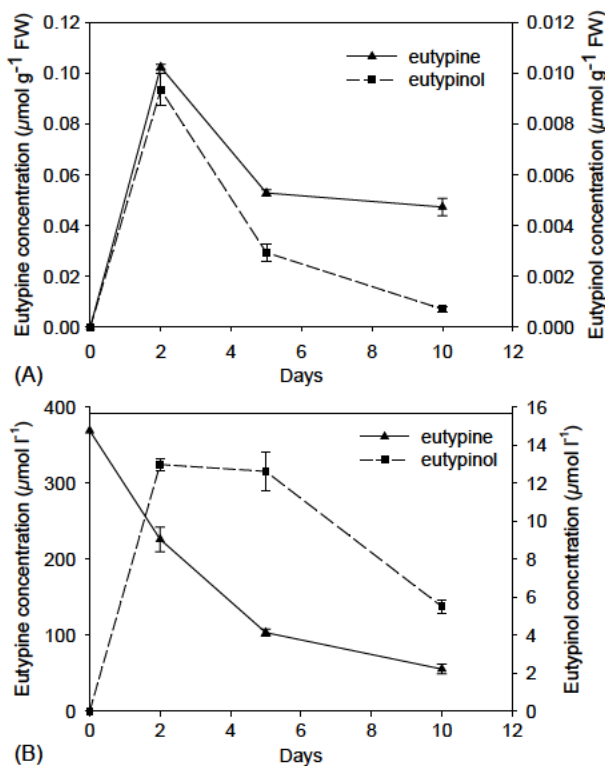


Fig. 7. (A) Eutypine and eutypinol concentrations in Ugni-blanc grape micro-cutting tissues cultured on 500 μM eutypine in the long experiment. (B) Eutypine and eutypinol concentrations in Gamay culture media after 2, 5 and 10 days of culture of the grape micro-cutting.

Plantlets were collected after 2, 5 and 10 days and divided into three parts, each one containing two leaves, starting with the basal part. Eutypine and eutypinol were detected in all levels after extraction. The highest eutypine concentration was observed in the first level 0.09, 0.08 and 0.05 $\mu\text{mol g}^{-1}$ FW on days 2, 5 and 10, respectively (Fig. 8A). Eutypine concentration was 0.02, 0.01 and 0.006 $\mu\text{mol g}^{-1}$ FW in the second level and 0.01, 0.003 and 0.004 $\mu\text{mol g}^{-1}$ FW in the third level, on days 2, 5 and 10, respectively, showing a decrease from the first to the third level. Eutypinol was detected after 2 days of culture in all the plantlet parts and then decreased in all the parts until day 10. The eutypinol concentration also decreased from the first level towards the third level (Fig. 8B). Eutypine and eutypinol peaks observed by HPLC analysis of the extracts of different plantlet parts were confirmed by LC-MS analysis (eutypine, $[M - H]^- = 185$; eutypinol, $[M - H]^- = 187$).

Experiments on grape micro-cuttings and plantlets showed no difference in their ability to transform eutypine. This means that leaves do not have any effect on eutypine uptake, which crosses the tissues following a concentration gradient from the highest to the lowest concentration. Previous in vitro studies have shown that eutypine penetrates grapevine cells through passive diffusion and its accumulation in the cytoplasm has been explained by an ion-trapping mechanism related to the ionisation state of the molecule [17].

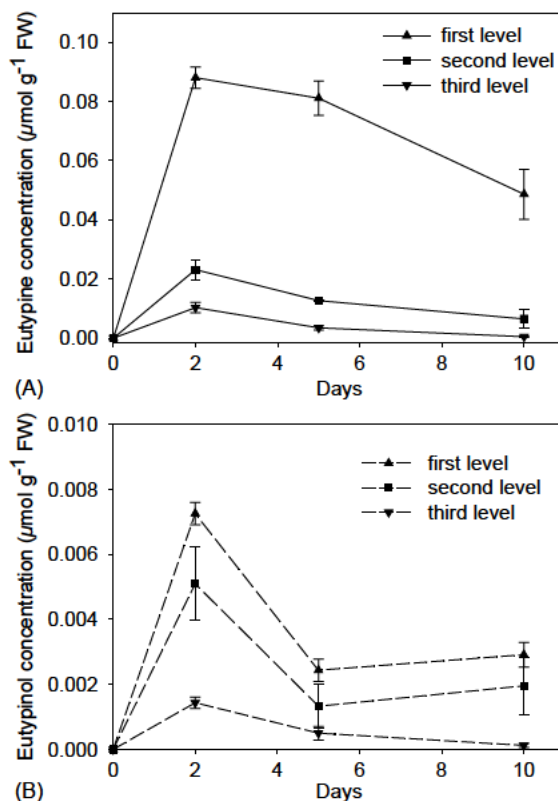


Fig. 8. Time-course of eutypine transport and eutypinol production in the different levels of grape plantlets cultured in the presence of 500 μM eutypine after 2, 5 and 10 days of culture. (A) Eutypine, (B) eutypinol.

Different experiments revealed that eutypine toxicity is an active process. It involves an increase in the rate of the eutypine biotransformation resulting in a high eutypinol concentration a short time after the beginning of culture. Results showed that grapevine tissues are able to transform eutypine within 1–48 h leading to high eutypinol concentrations in the different tissues. It may be hypothesised that the grapevine cells protect themselves in the first hours following the treatment by a rapid transient eutypine biotransformation into eutypinol. In the different grape tissues, the rapid decrease of eutypinol concentration after 48 h of culture means that eutypinol was metabolised. Such rapid transient response to fungal toxins was also observed in a previous study on *Pinus banksiana* suspension culture cells 12–24 h after treatment with a toxin prepared from ectomycorrhizal fungus [18]. An accumulation of specific methanol-extractable compounds and the induction of lignification for protection were found in the cells through the enhancement of the activities of three enzymes in the phenylpropanoid metabolic chain.

4. Conclusion

Our results indicate that in the in vitro culture incubation conditions used here, eutypine is photostable. Moreover, the agar concentration in the culture medium influences the absorption of eutypine by Gamay callus tissues. Eutypine

penetrated grapevine cells quickly and was then metabolised very rapidly into eutypinol. Eutypinol is excreted into the culture medium and also metabolised into other compounds. The use of micro-cuttings and in vitro plants showed that eutypine circulates in the plant and accumulates strongly in the bottom part of the stem. Moreover, leaves and stems acted similarly in the accumulation and metabolisation of eutypine.

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References

- [1] G.P. Munkvold, J.A. Duthie, J.J. Marois, *Phytopathology* 84 (1994) 186.
- [2] R. Tabacchi, *Pure Appl. Chem.* 66 (1994) 2299.
- [3] J. Fallot, C. Deswarte, S. Dalmayrac, C. Colrat, J.P. Roustan, *CR Acad. Sci. Paris* 320 (1997) 149.
- [4] J.M. Renaud, G. Tsoupras, R. Tabacchi, *Helv. Chim. Acta* 72 (1989) 929.
- [5] P. Tey-Rulh, I. Philippe, J.M. Renaud, G. Tsoupras, P. de Angelis, J. Fallot, R. Tabacchi, *Phytochemistry* 30 (1991) 471.
- [6] C. Deswarte, J. Eychenne, J. Davy de Virville, J.P. Roustan, F. Moreau, J. Fallot, *Arch. Biochem. Biophys.* 334 (1996) 200.
- [7] B.E. Amborab  , P. Fleurat-Lessard, J. Bonmort, J.P. Roustan, G. Roblin, *Plant Physiol. Biochem.* 39 (2001) 51.
- [8] S. Colrat, C. Deswarte, A. Latch  , A. Kla  b  , M. Bouzayen, J. Fallot, J.P. Roustan, *Planta* 207 (1999) 544.
- [9] E. Defrancq, R. Tabacchi, *J. Labelled Comp. Radiopharm.* 31 (1992) 1057.
- [10] C. Ambid, M. Moissef, J. Fallot, *Physiol. V  g.* 21 (1983) 82.
- [11] O. Souli  , J.P. Roustan, J. Fallot, *Vitis* 32 (1993) 243.
- [12] T. Murashige, F. Skoog, *Physiol. Plant* 15 (1962) 473.
- [13] I. Philippe, Ph.D. Thesis, INP Toulouse, Ecole Nationale Sup  rieure d'Agronomie, 1991.
- [14] F. Smit, I.A. Dubery, *Phytochemistry* 44 (1997) 811.
- [15] I.A. Dubery, V. Slater, *Phytochemistry* 44 (1997) 1429.
- [16] I.A. Dubery, L.G. Teodorczuk, A.E. Louw, *Mol. Cell Biol. Res. Commun.* 3 (2000) 105.
- [17] C. Deswarte, H. Canut, A. Klaebe, J.P. Roustan, J. Fallot, *J. Plant Physiol.* 149 (1996) 336.
- [18] M.M. Campbell, B.E. Ellis, *Planta* 186 (1992) 409.