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# Phytotoxins from fungi of esca of grapevine

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**Summary.** The chemical composition of the culture media of five fungi involved in esca of grapevine have been investigated. *Eutypa lata,* extensively studied some years ago, produces eutypine [4-hydroxy-3-(3-methyl-3-butene-1-ynyl)benzaldehyde] that seems to be the most phytotoxic metabolite. Using the same analytical method for *Stereum hirsutum*, we isolated nine compounds. Sterehirsutinal, a compound similar to frustulosine, but having two vinyl-acetylenic chains, possesses a toxicity comparable to eutypine. From *Phaeoacremonium chlamydosporum* we isolated nine metabolites that have not been extensively tested, but the simplest one, p-hydoxybenzaldehyde, shows marked toxicity. Other compounds (naphthalenone derivatives) are known to be involved in different wood diseases. From *Phaeoacremonium aleophilum* we also isolated p-hydroxybenzaldehyde and scytalone. Preliminary results on *Fomitiporia punctata* confirmed again the presence in the culture medium of p-hydroxybenzaldehyde and of a new chromanone biogenetically related to eutypine. The presence of molecules carrying the aldehyde function seems to play an important role in the toxicity of the five fungi implicated in esca.

**Key words:** esca, grapevine, phytotoxins, *Stereum hirsutum, Phaeoacremonium chlamydosporum, P. aleophilum, Fomitiporia punctata.*

## Introduction

The biological aspects of esca have been extensively investigated during the last decade in several grape-growing countries in Europe and elsewhere.

Contrary to other fungal wood diseases, which are caused by a single pathogen, esca results from the association of different fungi that can act in a concomitant association or in a succession of several microorganisms in different parts of the woody tissues.

Mugnai *et al*. (1999) began by considering esca as a single but complex disease and attempted to identify the action of the three fungi that are most commonly found in the woody tissues of esca-affected vines: *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*. A second hypothesis was that esca was an association of microorganisms acting individually in the wood decay process and was therefore not a simple disease, but a combination of diseases.

In addition, depending on the country and the authors that isolated and identified the microorganisms involved in esca, two other fungi, *Eutypa lata* and *Stereum hirsutum* have often been mentioned in this connection, particularly in France (Larignon and Dubos, 1997).

It is well known that fungi produce toxins in infected plants as well as in culture medium. Toxins, however, are extremely poisonous substances and are effective at very low concentration. Toxins injure host cells by affecting the cell membrane, the cellular transport system, or by inactivating, inhibiting or interrupting enzymatic reactions. Several toxic secondary metabolites produced by

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plant pathogens have been shown to elicit all, or part of the disease symptoms, also on other species of plants that are not normally attacked by the pathogen in nature.

The chemist considers the pathogen as a chemical reactor where, via biochemical and enzymatic reactions, secondary metabolites are produced. Among these molecules, some can be toxic to the plant. The isolation process from a culture medium has to be driven by a broad spectrum of phytotoxicity bio-assays.

Based on the chemical structure, these toxins can be simple molecules having a carbon skeleton, one or more chemical functions and low molecular weight (max. 500 uma), or complex molecules, having a higher mol. wt (up to many thousand Da), e.g. polypeptides: cerato-ulmin (duch elm disease) or fimbriatan and cerato-platanin (canker of the plane tree)

In a complex of molecules, some can act together and the combined toxicity can be higher than the sum of the toxicities of the individual molecules (synergism).

Our research focused mainly on small molecules, because they can be easily transported in the plant and can interact directly with complex molecules like polypeptides, proteins and polysaccharides.

In the case of esca, the problem is complicated and the chemical approach is more difficult because of the presence of many pathogens producing toxic syndromes.

For simplicity sake we assumed that each fungus produces individual toxins and we tried to investigate the corresponding culture filtrate separately.

## Materials and methods

Isolates of *S. hirsutum*, *P. chlamydosporum*, *P. aleophilum* and *F. punctata* were collected from different places by Larignon (INRA, Bordeaux, France) and Minervini (University of Milan), and grown on potato-dextrose-agar (PDA). Liquid cultures were made either on a broth prepared according to Eriksson and Petersson (1975) (*Phaeoacremonium*) or on carrot-broth *F. punctata*. The fungi were grown for four weeks at 25°C under normal light conditions. The mycelium was removed by filtration on Celite and the liquid phase extracted with ethyl acetate. The organic phase was concentrated under vacuum and the residue purified by column chromatography (silica gel; hexane /EtOAc gradient).

Several fractions of increasing polarity were collected. Screening of the mild-polar fractions led to the isolation of phytotoxic compounds. Further purification was achieved by semi-preparative HPLC on RP-C-18 column (MeOH – $H_2O$ gradient).

Biological activity of the fractions thus separated was based on the evaluation of the toxicity by visual estimation of withering, or weight loss after immersion of a cutting of young tomato plants, cv. Bonny Best (5–8 cm, 200–300 mg, two leaves) into a solution of about 0.5 to 1 mg $ml^{-1}$ of tested mixture, or pure substances, for 8 to 24 h. In all cases blank tests were carried out in the same conditions. In order to increase the solubility of the pure compounds, 5% of methanol (perfectly tolerated by the plants) were added to both the metabolites and the blank tests.

More specific tests on the most toxic metabolites were carried out at the Laboratory of Moët and Chandon (Epernay, France), and at ENSAT Toulouse (France):

– on living protoplasts (from hydroponic culture of *Vitis vinifera* cv. Cabernet Sauvignon, or Ugni blanc) by calculation of the percentage of surviving protoplasts after 24 h in presence of the compounds at the concentration of $10^{-5}$-$10^{-6}$ M.

– on callus (from *Vitis vinifera* cv. Gamay) grown in standard culture media in presence of the toxin at different concentrations (100 μM, 250 μM and 500 mM) for 28 days. The growth of callus tissue was taken to be the weight of the callus, and expressed as a percentage of callus tissue weight when cultivated without the presence of toxins.

Structural elucidation of the isolated and purified compounds was performed by spectroscopic analysis, using UV, FT-IR, high field (200 and 400 MHz) $^{1}$HNMR and $^{13}$CNMR, EI-, CI- and ESI-MS.

## Results

More than 10 years ago we investigated *E. lata*, the agent of the eutypa dieback (dying arm disease). From the culture filtrates of this pathogen we isolated a family of vinylacetylenic compounds

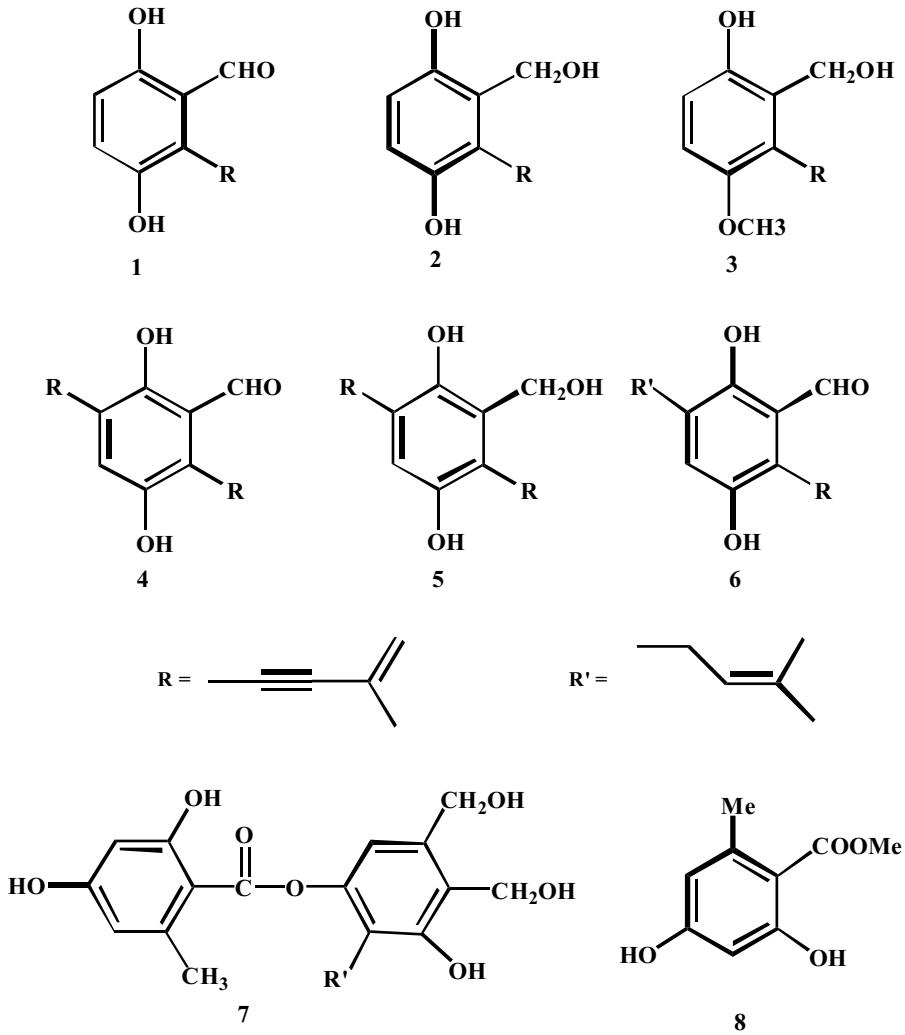



Fig. 1. Compounds isolated from *Stereum hirsutum*.

(Renaud *et al*, 1989). One of those, eutypine, showed extreme toxicity to grapevine. Using a very sensitive analytical method, MS-MS, we demonstrated the presence of the toxin in the rising sap, the branches, the leaves, and the inflorescences. The simple reduction of the aldehyde function (eutypine to eutypinol) is sufficient to detoxify and block the action of eutypine. Colrat *et al*. (1999) (ENSAT, Toulouse) demonstrated that the cv. Merlot (resistant to eutypa dieback) is able to perform this reaction by a specific reductase enzyme (ERE).

Recently Molyneux (1999) reported that methoxy-siccayne is also a toxic metabolite of *E. lata*.

Using the method described above, we investigated *S. hirsutum* (Dubin *et al.*, 2000). We isolated a known compound, hirsutic acid, and eight major compounds (Fig. 1). Sterehirsutinal (**4**), a compound similar to frustulosine (**1**), having two vinyl-acetylenic chains, possessed a high toxicity comparable to eutypine, showing 50%, 80%, and 100% inhibition of the callus growth at 100 μM, 250 μM and 500 μM concentrations respectively.

On tomato plants, withering appeared at concentrations of 1-3 mM. Compared to eutypine, sterehirsutinal causes a rapid and total disgregation of stems dipped into the solution. The protoplast tests confirmed these results.

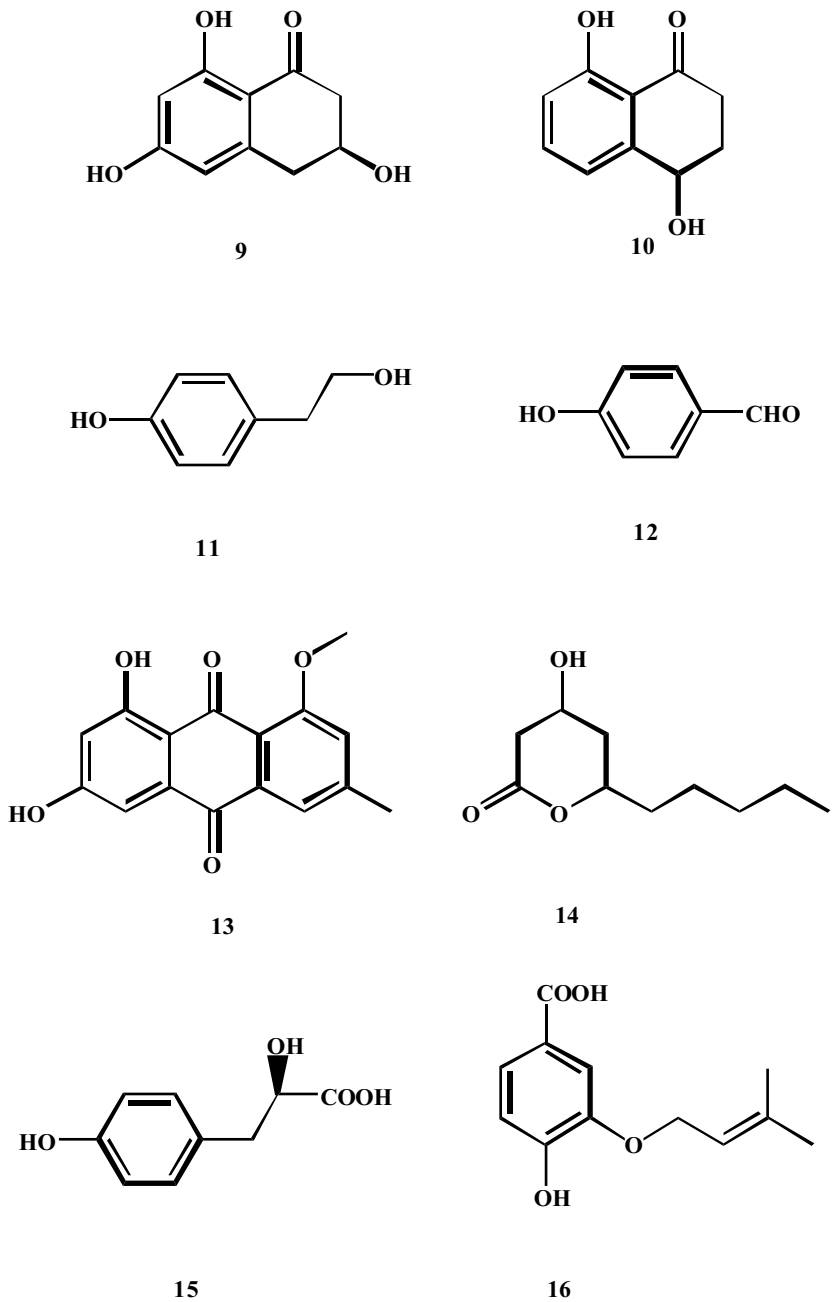
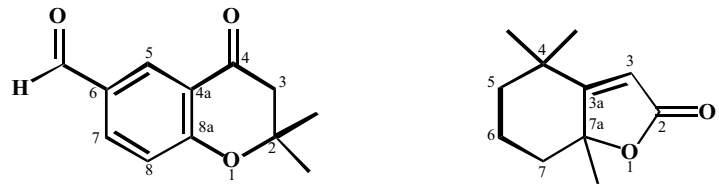



Fig. 2. Compounds isolated from *Phaeoacremonium chlamydosporum*.

We suppose that sterehirsutinal is not transported into the vessels. Indeed, the analysis (using ESI-LC-MS$^n$) of the rising sap, or leaves, did not demonstrate the presence of the toxin. In contrast, sterehirsutinal was present in all the samples of decayed wood.

In order to confirm the structures and to have enough material for the biological tests and action mechanism, we prepared synthetically sterehirsutinal, sterehirsutinol (Fkyerat *et al*., 1999), methylfrustolosine and methylfrustulosinol. The eutypine reductase enzyme (ERE) reduced the aldehyde



**17** **18**

Fig. 3. Compounds isolated from *Fomitiporia punctata*.

function of compounds **1** and **4** to the corresponding alcohols **2** and **5**.

From *P. chlamydosporum*, we isolated nine metabolites (Fig. 2) that have not yet been extensively tested, but the simplest one, p-hydoxy-benzaldehyde, shows an inhibition of 20% at 100 mM and 100% at 1mM. Acids **15** and **16** are also active on protoplasts at 1mM concentration. Other compounds, **9** (scytalone), **10** (isosclerone) and **11** (tyrosol) are known to be involved in different wood diseases. The acid **16** and the corresponding aldehyde have been synthesized for further biological assays.

We did not investigate the chemical composition of the aqueous phases and the mycelium that could contain exopolysaccharides (pullulans), previously stated by Sparapano *et al.*, (1998) to be phytotoxic metabolites of the two species of *Phaeoacremonium*.

From *P. aleophilum* we also isolated p-hydroxybenzaldehyde (**12**) and scytalone (**9**).

Preliminary results on *F. punctata* confirmed the presence in the culture filtrate of p-hydroxybenzaldehyde and of a new chromanone, biogenetically related to eutypine (Fig. 3).

Dihydroactinolide (**18**) is a known terpene derivative that can arise from the culture medium.

6-Formyl-2,2-dimethyl-4-chromanone is a new natural compound, biogenetically related to eutypine. The synthesis and the bioactivity evaluation of this compound are in progress.

## Discussion

A global analysis of the results obtained until now, of the different metabolites isolated from culture filtrates of the five microorganisms involved in esca, showed the presence of at least one phytotoxin in each culture:

| Microorganism | Toxin |
|---|---|
| *E. lata*: | Eutypine |
| *S. hirsutum*: | Sterehirsutinal |
| *P. chlamydosporum*: | 4-Hydroxy-benzaldehyde |
| | 3-(3-methyl-but-2-enyloxy-4-hydroxy)benzoic-acid |
| *P. aleophilum*: | 4-Hydroxy-benzaldehyde |
| *F. punctata*: | 4-Hydroxy-benzaldehyde |
| | 6-Formyl-2,2-dimethyl-4-chromanone |

The analysis of the chemical structure of these toxins shows:
– the aldehyde function, on an aromatic ring in the *ortho* or *para* position, is always present (in one case it was oxidized);
– the common biogenetic precursor of all the toxins could be 4-hydroxy-benzaldehyde;
– compound **16** [3-(3-methyl-but-2-enyloxy)-4-hydroxybenzoic acid] corresponds to the oxidized form of an aldehyde possessing a structure similar to that of eutypine;
– the chromanone derivative **17** could also be biogenetically related to eutypine.

In our opinion, the production of hydroxy-benzaldehyde derivatives plays an important role in the toxicity of the five fungi implicated in esca. The hydroxy substituent on the aromatic ring, and even more the presence of a vinylacetylenic chain, contributed strongly to the increase in phytotoxicity.

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