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Do fungal naphthalenones have a role in the development of esca symptoms?

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Summary. As part of a study on fungal toxins, we isolated and identified a series of naphthalenone-related compounds derived from five species of pathogenic fungi. We report here the investigation of the liquid culture medium of *Phaeoacremonium aleophilum*, one of the most important pathogens in the first stage of esca. Various biological assays on grapevine callus and on *Arabidopsis thaliana* were performed to ascertain the toxic effect of the seven naphthatlenones isolated from this pathogen.

Key words: esca symptoms, *Phaeoacremonium aleophilum*, toxicity.

Introduction

Several pathogenic fungi associated with wood and trunk diseases of plants produce fungal melanin, a high-molecular-weight black pigment. Melanin not only protects the fungus but also plays an essential role in the manner in which the ascomycetes, basidomycetes and deuteromycetes penetrate the cell wall of the plant (Haword *et al*., 1989). Melanin is synthesised by the polymerisation of 1,8-dihydroxy-naphtalene (DHN) (Bell *et al*., 1976). The DHN-melanin biosynthesis pathway (Fig.1), as described by Wheeler and Bell (1988) is based on genetic and biochemical evidence obtained from *Verticillium dahliae* (Wheeler, 1981; 1982) and *Wangiella dermatidis* (Geis *et al*., 1984; Wheeler, 1985). Initially it was thought that the acetyl-coenzyme A was the precursor for the pathway. However, Fujii *et al*. (2000) demonstrated that, at least for *Colletotrichum lagenarium,* malonyl-CoA serves as the starter and extender unit for the polyketide synthase PKS1, catalysing the first step in the biosynthesis pathway (Adachi, 1998). The polyketide synthase (PKS) converts malonyl-CoA to the first detectable intermediate of the pathway, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). Following this, the 1,3,6,8-THN is reduced by a specific reductase enzyme to produce scytalone (Alspaugh *et al*., 1997).

From different cultures of plant pathogenic fungi, including those of *Ceratocystis fimbriata* f. sp. *coffea* (Grémaud, 1996), *C. f.* f. sp. *platani* (Burki, 2003), *Ophiostoma ulmi* (Michel, 2002), *Stagnospora* sp. (Nicolet *et al.*, 1999) and *Phaeomoniella chlamydospora* (Tabacchi, 2000), we isolated several naphthalenone-related compounds (Table 1). All isolated metabolites were derived from 1,3,6,8-tetrahydroxynaphthalene (T4HN)[1] and are thought

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[1] Abbreviations. DHN, dihydroxynaphthalene; HJ, hydroxyjuglone; THT, trihydroxytetralone; THN, trihydroxynaphthalene, T4HN, tetrahydroxynaphthalene, HS, hydroxyscytalone.

Table 1. Naphthalenone derivatives isolated from fungi with the corresponding biological assays.

Compounds	Fungus (origin)	Biological assay[a]
Vermelone	*Ceratocystis fimbriata* f. sp. *coffea* *Stagnospora convolvuli*	L, C
Scytalone	*Ceratocystis fimbriata* f. sp. *platani* *Stagnospora* sp. *Ceratocystis fimbriata* f. sp. *coffea* *Phaeomoniella chlamydospora* *Phaeoacremonium aleophilum*	L, C, A,
Cis-1(2H)-3,4dihydro-2,4,8-THT	*Ceratocystis fimbriata* f. sp. *platani*	L,A, C
Trans-1(2H)-3,4dihydro-2,4,8-THT	*Ceratocystis fimbriata* f. sp. *platani*	L, A, C
Trans-1(2H)-3,4dihydro-3,4,8-THT	*Ceratocystis fimbriata* f. sp. *coffee* *Stagnospora* sp.	L, A
Cis 4-hydroxy scytalone	*Ceratocystis fimbriata* f. sp. *platani* *Ceratocystis fimbriata* f. sp. *coffea* *Phaeoacromonium aleophilum*	L, C
Trans 4-hydroxy scytalone	*Ceratocystis fimbriata* f. sp. *platani*	L, C
3-methyl scytalone	*Stagnospora* sp.	
Isosclerone	*Phaeomoniella chlamydosporum* *Phaeoacromonium aleophilum*	L A, C

[a] L, leaf; C, vine callus; A, *Arabidopsis*.

to be precursors in the pathway of fungal DHN-melanin (Fig. 1). In a recent study, we investigated a liquid culture of *Phaeoacremonium aleophilum,* a pathogen involved in the first stage of esca. Seven naphthalenones were isolated and their structure elucidated (Fig. 1). We report here on the compounds that were isolated and on their biological activity against grapevine callus and the model plant *Arabidopsis thaliana*. In the light of the study findings on a possible active role for naphthalenones inside infected plants are discussed.

Materials and methods

Organisms and culture media.

The isolate of *P. aleophilum* PP_2SO_{24} was collected by Philippe Larignon (INRA, Bordeaux, France) from Montbazillac in 1996 and maintained on a PDA medium at 4°C . The fungus was grown on Czapek agar plates for 10 days at 25°C, before inoculation into 20×500 ml Erlenmeyer flasks each containing 150 ml of Czapek medium supplemented with 0.1% yeast and 0.1% malt extract (pH 5.8). The cultures were incubated at 25°C and shaken in the dark for 28 days.

Extraction and compounds purification.

Culture filtrate (3 l) was extracted with ethyl acetate, then acidified at pH 2 with 1 M CH_3COOH and extracted again with ethyl acetate. Extracts were concentrated separately under vacuum. The different metabolites were isolated on a reverse-phase C18 column by high performance liquid chromatography using a gradient of methanol in water. The neutral extract (113 mg) yielded scytalone (8 mg), isosclérone (6 mg) and 4-hydroscytalone (4-HS) (0.8 mg), 2,4,8-trihydroxytetralone (2,4,8-THT) (1.1 mg), 3,4,8-trihydroxytetralone (3,4,8-THT) (0.8 mg), 1,3,8-trihydroxynaphthalene (1,3,8-THN) (0.6

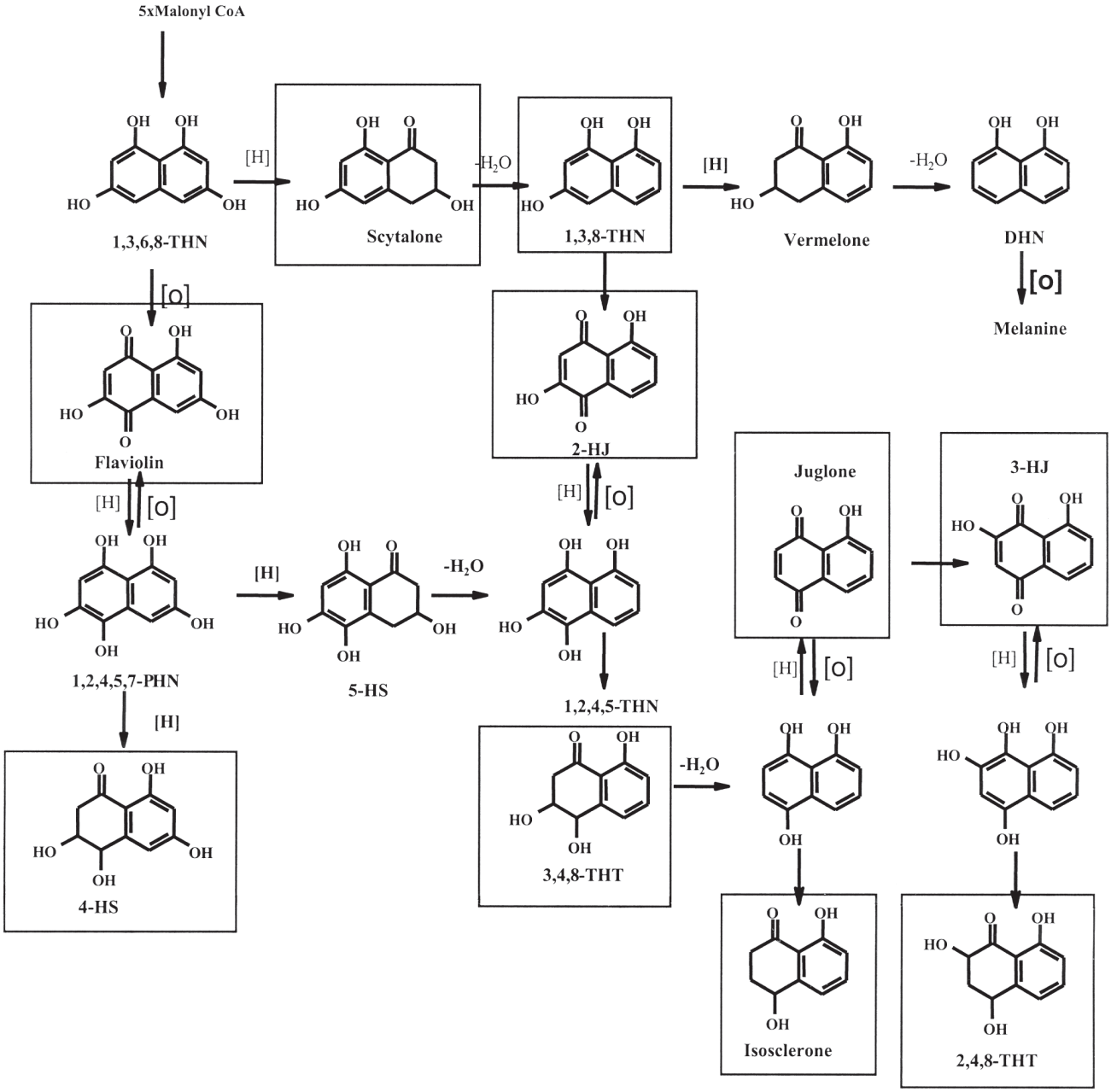

Fig. 1. The pentaketide pathway of melanin synthesis with flaviolin and 2-HJ branch pathways redrawn after Wheeler (1985) highlighting the isolated molecules.

mg). However, the main compounds in the acidic extract (215 mg) were flaviolin (8 mg) and traces of 2-hydroxyjuglone (2-HJ).

Biological assays

For the assays, juglone was purchased from Fluka (Fluka Chemie, Gmbh, Buchs, Switzerland), 2-hydroxyjuglone and 3-hydroxyjuglone were synthesised according to Thomson (1951) and McLeod (1960). Enough material to perform the bioassays of 2,4,8 and 3,4,8-trihydroxytetralone was obtained by synthesis carried out as described by Couché *et al.,* (2003).

The activity of the crude extracts was evaluated by measuring their necrotic effect on a disc of vine leaves (Sugawara, 1985) at 500 mg ml^{-1} and 250 mg ml^{-1}.

Pure compounds were tested on grapevine callus kindly supplied by Roustan (INRA, Toulouse, France). Callus cultures of *Vitis vinifera* cv. Gamay were cultivated on a growth medium according to Ambid *et al.,* (1983) in a 12-h day for 28 days at 28°C. Calli were weighed before and after incubation and percent growth was calculated.

Arabidopsis thaliana assay.

Seeds of the *A. thaliana* ecotype Columbia were surface-sterilised in 5% sodium hypochloride for 10 min, and sown on agar medium (2.15 g l^{-1} Murashige and Skoog [1962] basal salts; 0.5 g l^{-1} MES buffer; 7 g l^{-1} phytoagar; pH 5.7). Seeds were incubated in a phytotron growth chamber at 22°C under an 8-h day. The plates were placed vertically, so that the roots grew downwards along the surface of the agar medium. Root length was measured daily for three days and the final measurement, shown in Fig. 4, was made after eight days of growth. The percentage growth, relative to the control growing on media with no toxin added, was calculated.

Results and discussion

In previous studies carried out in our laboratory on plant trunk pathogenic fungi, nine melanin intermediates were isolated and identified by ^{1}H NMR and MS techniques and were subjected to the biological assays described above. A list of the compounds and assays performed is given in Table 1.

Seven compounds (Fig. 1) were isolated from liquid culture of *P. aleophilum* and identified with NMR and ESI-MS (negative mode). Scytalone m/z 193 $(M-H)^-$, isosclerone m/z 177 $(M-H)^-$, 4-hydroxyscytalone m/z 209 $(M-H)^-$, 2,4,8-trihydroxytetralone m/z 193 $(M-H)^-$, 3,4,8-trihydroxytetralone m/z 193 $(M-H)^-$, 1,3,8-trihydroxynaphhtalene m/z 175 $(M-H)^-$ and flaviolin m/z 205 $(M-H)^-$. Spectroscopic data were in agreement with those reported in the literature for these compounds (Davies, 1954; Findlay, 1973; Aldridge, 1974; Morita, 1974; and Bell, 1976; Yasuo, 1985).

Scytalone and isoscleronee were reported by Evidente *et al.* (2000) to be the main compounds isolated from cultures of *P. aleophilum*. These authors indicated that scytalone at 0.05 mg ml^{-1} and isoscleronee at 0.1 mg ml^{-1} induced symptoms on the foliar lamina 6–8 h after absorption. In our callus assay (Fig. 2), there was hardly any growth inhibition; indeed, there was actually an increase of growth at low concentrations (0.1 mM) of scytalone and 3,4,8-THT, and at 0.25 mM for isosclerone. Furthermore, scytalone at 0.3 and 0.01 mM and 3,4,8-THT at 0.01mM promoted growth of the roots of *Arabidopsis* (Fig. 4). These results are in agreement with the biological assays on isosclerone in Morita (1974), where it was found that isosclerone at a concentration of 1≈10 ppm stimulated the root elongation of rice seedlings by ca. 30%. Findlay (1973) however reported that neither isosclerone nor scytalone had significant antifungal activity.

On the other hand, naphthoquinones such as juglone, 2-HJ and 3-HJ inhibited callus growth at 0.1 mM, and flaviolin at 0.25 mM (Fig. 3); like juglone, 2-HJ and 3-HJ reduced root growth of *Arabidopsis* (Fig. 4).

According to these biological results, the isolated metabolites can be divided into two classes: tetralones such as scytalone, isoscleronee, 2,4,8-THT and 3,4,8-THT, which promote callus growth; and naphthoquinones like juglone, 2-HJ, 3-HJ and flaviolin, which inhibit growth.

In a previous study (Bürki *et al.,* 2003), it was found that 2,4,8 and 3,4,8-THT tested on plane leaves were not active for the first 48 h. However, HPLC analysis of the remaining solution for the following three days confirmed that 2,4,8 and 3,4,8-THT were then oxidised into 2-HJ and 3-HJ. When the same test was performed with 2-HJ and 3-HJ synthesised from juglone, marked necrosis was

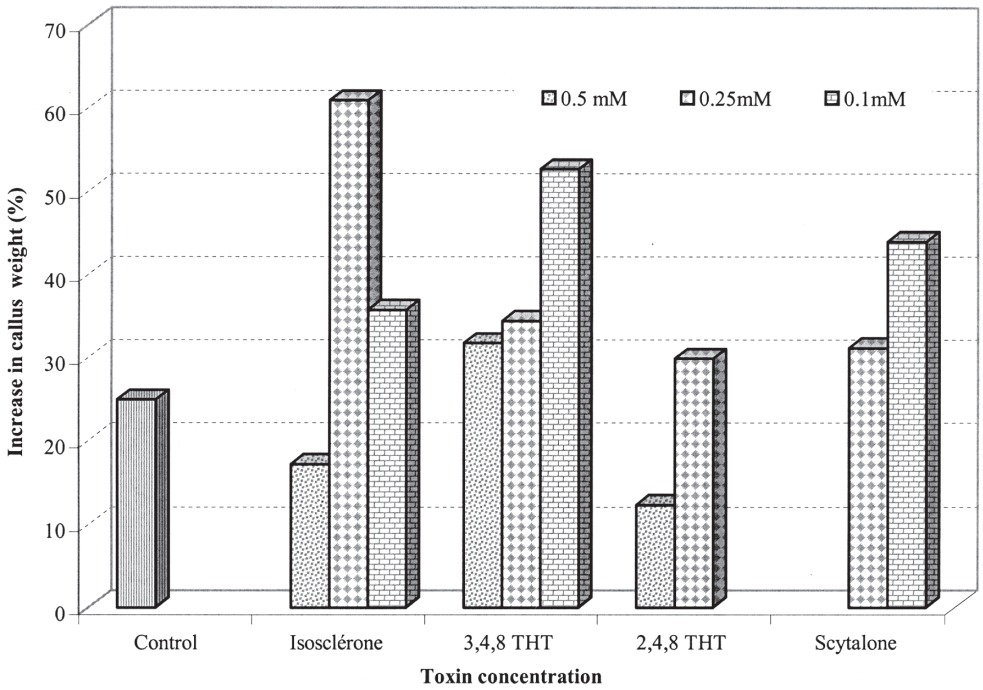

Fig. 2. Effect on the growth of grapevine cv. Gamay callus of the presence of different concentrations of tetralone (3,4,8 THT and 2,4,8 THT) in comparison with scytalone and isosclerone. (Concentrations 0.1 mM of 2,4,8 THT and 0.5 mM of scytalone were not tested).

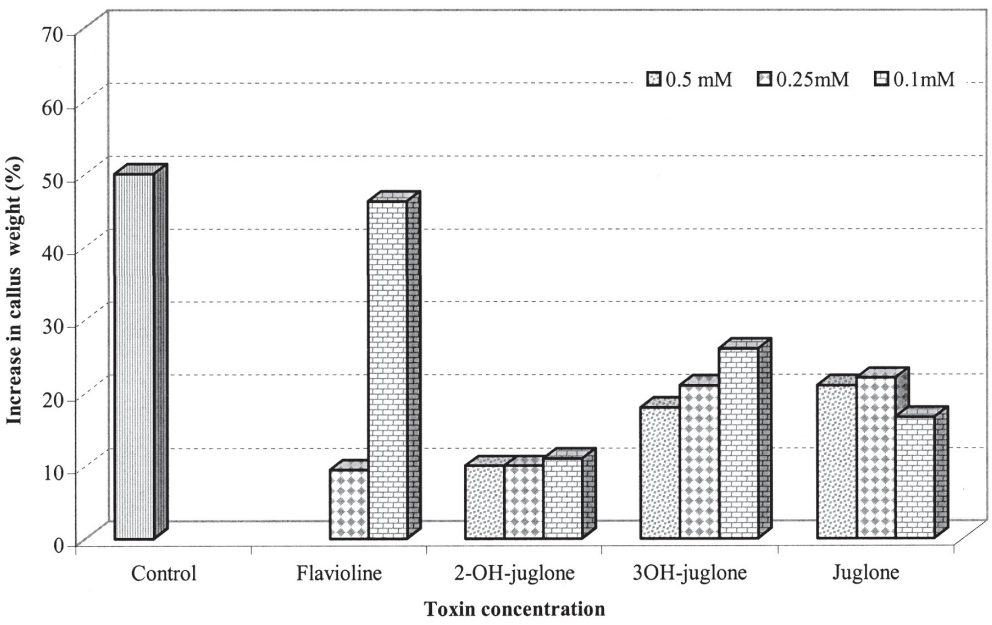

Fig. 3. Effect on the growth of grapevine cv. Gamay callus of the presence of different concentrations of naphthoquinones (2-OH-juglone and 3-OH-juglone) in comparison with flavioline and juglone. (Flaviolin was not tested at 0.5 mM).

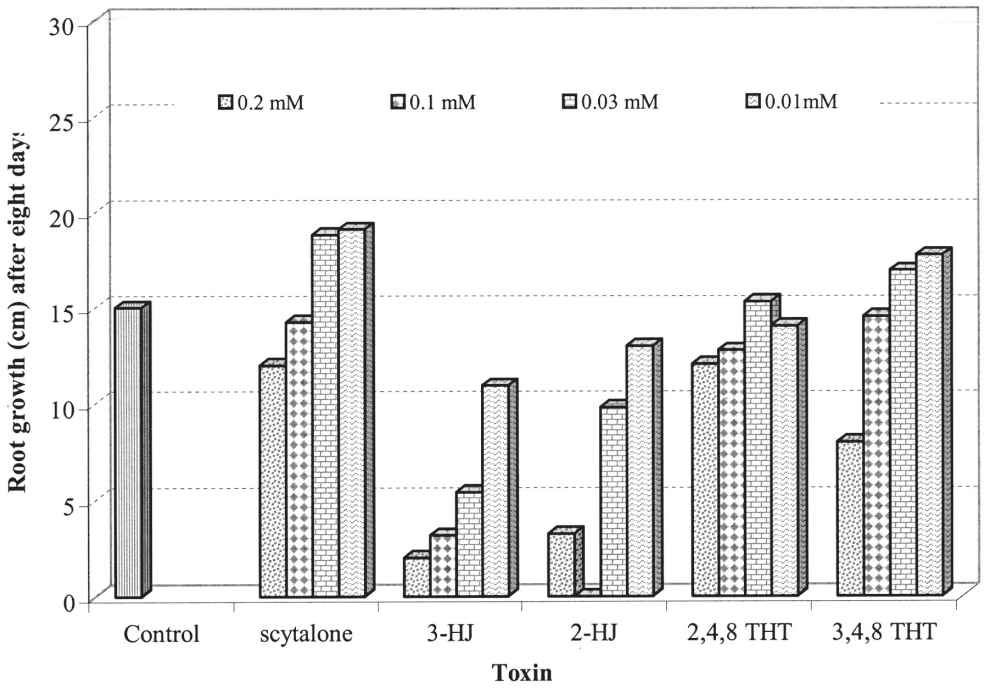

Fig. 4. *Arabidopsis thaliana* root growth after 8 days of incubation in the presence of various toxins at four different concentrations. (2HJ at 0.1 mM was contaminated).

produced on the plane leaves in less than 8 h. With the *A. thaliana* assay, 2,4,8-THT and 3,4,8-THT did not inhibit root growth, but 2-HJ and 3-HJ inhibited 80% of root growth. These results are in agreement with Yamaguchi (1982) who found that 2-HJ was toxic and inhibited spore germination in *Pyricularia oryzae*. Iwasaki *et al.* (1972) isolated 3,4,8-THT and 4-HS from *P. oryzae* and reported that 3,4,8-THT reduced growth of rice seedlings at high concentration, but on the contrary slightly stimulated growth if the seedlings were pretreated with a 100 ppm solution of this same substance. These results are in agreement with the assay on *Arabidopsis* roots (Fig. 4). By contrast, 4-HS had no significant activity on the growth rate of rice seedlings.

Since 2-HJ is found in *P. aleophilum* culture (although 3-HJ is not), there is a possibility that 2,4,8-THT and 3,4,8-THT are oxidised into 2-HJ and 3-HJ in the plant. This possibility should be investigated and grapevine wood infected with *P. aleophilum* tested to confirm it.

As was mentioned (Bürki *et al.*, 2003), the naphthoquinones are cytotoxic and their cytotoxicity is attributed to the formation of a covalent adduct, through a Michael 1,4 addition between the quinone and a protein thiol or amino group (Oberth *et al.*, 1992). The biological function of scytalone, vermelone and 2-HJ branch products, if any, is not clear. Juglone is the most widely studied of these compounds. It is highly toxic to plants and animals (Harborne, 1982). In fungal cells, juglone generates superoxide radicals diminishing the cellular pool of reduced pyridine nucleotides which are particularly necessary for cells exposed to oxidative stress. It is well known that one of the primary responses of plants to phytopathogenic invasion is the activation of O_2^- and H_2O_2 generating processes (Scandalios, 1990; Dangl, 1996). Many plants also produce auto-oxidisable quinone defence compounds, which oxidise NAD(P)H in plant and phytopathogenic cells, with the formation of superoxide radicals and hydrogen peroxide (Thomson, 1971). In response to this plant defence the phytopathogenic fungi synthesise naphthoquinones, which possess a phytotoxic action and suppress the defence reactions (Medentsev, 1998).

Naphthoquinones lower the resistance of plants to phytopathogenic fungi and hence enhance fungal virulence. The production of naphthoquinone pigments appears to be an important

component of the disease process as these pigments act as non-specific virulence factors inhibiting the plant defence reaction (resulting in plant hypersensitivity).

It has been reported that actively melanising cell-like appressoria of *P. oryzae* accumulate melanin intermediates or their derivatives (phenols and quinones) in the presence of agents blocking melanin biosynthesis such as Tricyclazole® or Carpropamide® (Kurahashi, 1997). As phenols and quinones are highly toxic to living cells, it is assumed that the abnormal accumulation of such metabolites as 2-HJ bring about the self-destruction of the appressorial function of the fungus (Yumita, 1983).

The application of fungicides, which are systemic inhibitors of melanin biosynthesis, can control these fungal infections since they interfere with the melanisation of fungal cells. Examination of the biosynthetic pathway, however, indicates an interesting departure from the norm. The fungicide inhibits the infection by blocking melanisation, which leads to the accumulation of shunt metabolites (flaviolin, 2-HJ and 3-HJ) in the fungal cells (Fig. 1). But with *P. aleophilum*, it is the shunt-pathway metabolites themselves that are phytotoxic. Application of Tricyclazole® or Carpropamide® could actually increase the virulence of the fungus, by increasing the production of these toxins.

Comparable studies using plant material at very early stages of infection may prove more fruitful in discovering these naphthoquinones in plant tissue. Such knowledge would be helpful in more firmly establishing the role of naphthoquinones in the infection process. Moreover, studies on esca-infected grapevine with over-cropping symptoms would be helpful in establishing whether tetralones have any role in inducing these symptoms.

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