Structure and biological activity of Xanthyletin 
a new phytoalexin of Citrus.

AKHTAR J. KHAN, G. KUNESCH, S. CHUILON and A. RAVISE

INTRODUCTION

It is evident from different plant-fungus interaction studies that fungitoxic compounds of host origin (phytoalexins) produced during infection, play a role in defence reaction (FARKAS and KIRALY, 1962; KUC, 1972). The accumulation of phytoalexins is more prominent at the site of infection (ALBERSHEIM and VALENT, 1978; BALEY, 1982). The Citrus response to gummosis disease caused by Phytophthora spp. ranges from susceptibility to resistance (BOCGAS and LAVILLE, 1978; de VALLAVEIEILLE, 1983). In orchards, treatments with fosetyl Al [Aluminium tris (0-ethyl-phosphonate)] confer a protection against gummosis (LAVILLE and CHALANDON, 1982). During investigations on the defence reactions of the Citrus species against Phytophthora spp. (KHAN and RAVISE, 1985) we have identified one of the inhibitors. The purpose of the present investigation is the characterization of this phytoalexin whose synthesis occurs at high level in tolerant Rough Lemon and is triggered by fosetyl Al treatment in susceptible Valencia Late.

MATERIAL AND METHODS

Citrus seedlings, Rough Lemon tolerant and Valencia Late susceptible to gummosis (de VALLA VEILLE and PERRIER, 1981) were raised in green house at 25°C. 7 days old mycelial discs of P. citrophthora and P. parasitica (provided by Institut de Recherches sur les Fruits 'et Agrumes) were used for inoculation on stems of Valencia Late and Rough Lemon, respectively. After 4 weeks of incubation, phenolic substances were extracted in 90% methanol from root, shoot and leaves. The purification of fungitoxic compounds was carried out successively by preparative thick layer chromatography on silica gel H in hexane - ethyl acetate (1 : 1). Further purification was achieved by LH-20 gel column chromatography eluted

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with chloroform, and finally by high performance liquid chromatography (HPLC) on 5 μ silica column 3/8' x 30 cm eluted with hexane - ethyl acetate-methanol (50 : 50 : 5). The thin layer chromatography (TLC) data were obtained on silica plates in several solvent systems: hexane - ethyl acetate (2 : 1), hexane-ethyl acetate-methanol (60 : 40 : 3), chloroform-methanol (95 : 5) and chloroform (GOTTLIEB, 1972). The fluorescence of xanthyletin was observed under ultraviolet (U.V.) at 254 nm and 366 nm.

The coumarin was also visualised by spray reagents: diazotised p nitroaniline and antimony chloride at saturation in chloroform.

The evaluation of xanthyletin in tissue extracts was carried out by HPLC: gradient of organic solvents (Hx - Ae - Me) on silica column (5 μ - 3/8' x 30 cm) and gradient of methanol on silica C18 column (7μ - 3/8' x 30 cm) with U.V. detection.

The structure determination was carried by mass spectrometry (MS) with a G.S. - M.S. Nermag R10 - 10C coupled with a computer and by Nuclear Magnetic Resonance (NMR) with a Bruker apparatus 200 Hz in CDC13. The Infra Red (I.R.) spectrum was recorded on a Perkin-Elmer 397 in KBr.

The toxicity tests were carried out on TLC for Cladosporium cladosporioïdes inhibition (TAQUET, 1985) and in concavity slides for P. citrophthora (RAVISE and KIR-KIACHARIAN, 1976) using synthetic medium. The in- fluence of concentration between 25μg to 200μg/ml xanthyletin, the length of incubation period (25 to 120 hours) and the association with other phenolics, mainly 3,4-dimethoxy-benzaldehyde, were studied.

RESULTS

Not detected in tissues of uninoculated plants, Xanthyletin was accumulated in roots and shoots of Rough Lemon after inoculation by P. citrophthora. In the tissues of susceptible Valencia Late inoculated by P. parasitica the coumarin was detected at traces level: this synthesis was triggered by treatment with Fosetyl Al.

Chromatographic data.

We have characterized the coumarin by TLC in several solvent systems:

- hexane - ethyl acetate (2 : 1) Rf = 0,51
- hexane - ethyl acetate - methanol (60 : 40 : 3) Rf = 0,69
- chloroform - methanol (95 : 5) Rf = 0,74
- chloroform Rf = 0,30
- dichloromethane Rf = 0,48

Xanthyletin shows a strong absorption at 254 nm and intense fluorescence at 366 nm.

The HPLC data are:

- on C-18 column with U.V. detection at 250 nm eluted between 60-70 % of methanol gradient
- on silica column with U.V. detection at 280 nm in a gradient of hexane (A) ethyl acetate-methanol (10 : 1) B, eluted between 50-60 % of eluent B.

Spectrometric data:

The U.V. spectrum of coumarin in distilled methanol between 400-200 nm gives λ max. : 160 and 340 nm; λ min. : 250 and 290 nm.

Structure determination.

The NMR spectrum of xanthyletin in CdCl3 at 200 MHz (Figure 1) showed clearly the presence of two methyl groups at = 1,45 ppm (singlet), two signals corresponding to two protons which can be attributed to a double bound conjugated to a carbonyl group. Two further doublets (J = 10 Hz) as well as two singlets of one proton each at 6,70 and 7,03 ppm suggested the presence of a dimethyl chromene unit on an aromatic ring possessing two protons in para position. The above data strongly favored as structure a coumarin with an aneled dimethyl chromene ring. This was further confirmed by a carbonyl absorption in the Infra Red at 1720 cm⁻¹ (in KBr).

The mass spectra in the Champ Ionisation mode indicated a molecular weight of 228 in agreement with a molecular formula of C14 H12 O3 (Figure 2). Comparison of above data with known physical constants of xanthyletin (STECK, 1971) definitely confirmed the structure determination.

Fig. 2 • STRUCTURE OF XANTHYLETHIN (C14 H12 O3).

Toxicity test.

The growth of Cladosporium cladosporioides was not inhibited with 100 μg coumarin on TLC plates. Spots of inhibition ranging 10-15 mm in diameter were observed with 200 or 300 μg concentration applied on plates. In concavity slides, the inhibition of growth of P. citrophthora begins at the concentration of 25μg/ml and the growth was completely inhibited with 75μg/ml during more than one week of incubation. 100 μg coumarin/ml was found lethal for the fungus. The results of toxicity of the diffe-
Fig. 1 • NMR SPECTRUM OF XANTHYLETHIN IN CDCl₃ (BRUKER, 200 MHz).
rent concentrations of inhibitor is summarised in table 1. The toxicity of coumarin at 50 μg/ml decreased with time. This concentration inhibited 90% mycelial growth during one day but after two, to five days of incubation the growth was respectively 33%, 43%, 50%, 75% as compared to the reference.

The inhibition of P. citrophthora by xanthyletin at 25 μg/ml was increased by several phenolic compounds mainly by 3,4-dimethoxybenzaldehyde at 50 μg/ml (table 2). The toxicity of coumarin at 50 μg/ml decreased after 24 hours of incubation, during the same period modification of structure occurred even in distilled water. According to data of TLC and U.V. spectra, we supposed that an oxidation occurred: two compounds appeared at Rf = 0.39 and 0.12 in dichloromethane instead of Rf = 0.48 for coumarin. The U.V. spectra recorded between 380 and 200 nm showed appearance of new peaks with λ min. = 275 nm, λ max. 329 nm, shoulder = 250 nm.

**TABLE 1 - Inhibition of mycelial growth of P. citrophthora in vitro by different concentrations of xanthyletin after different incubation periods-growth (μm, mean of 20 measurements).**

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>452</td>
<td>762</td>
<td>930</td>
<td>1156</td>
<td>1433</td>
</tr>
<tr>
<td>25</td>
<td>102</td>
<td>526</td>
<td>690</td>
<td>829</td>
<td>1160</td>
</tr>
<tr>
<td>50</td>
<td>52</td>
<td>217</td>
<td>395</td>
<td>562</td>
<td>904</td>
</tr>
<tr>
<td>75</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE 2 - Inhibition of mycelial growth of P. citrophthora by xanthyletin (25 μg/ml) or by 3,4-dimethoxy-benzaldehyde (50 μg/ml) alone or associated after different incubation periods.**

- growth (μm, mean of 20 measurements.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Xanthyletin</td>
<td>150</td>
</tr>
<tr>
<td>3,4-dimethoxy-benzaldehyde</td>
<td>156</td>
</tr>
<tr>
<td>Xanthyletin + 3,4-dimethoxy-benzaldehyde</td>
<td>0</td>
</tr>
</tbody>
</table>

The synergistic effect of this coumarin associated with other phenolic compounds is an indication of a complex reaction of Citrus against the pathogen depending on the host. Further investigations will improve the knowledge of these defence mechanisms and their stimulation by fosetyl Al treatment.

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Mechanisms of phytoalexin accumulation.

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