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

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SUMMARY - Xanthyletin, a coumarin, have been isolated from tissues of Citrus infected by *Phytophthora* spp. This compound is identified by Infra Red, Mass Spectrometry and Nuclear Magnetic Resonance data. It is an efficient inhibitor of *Phytophthora citrophthora in vitro* and a synergistic effect is observed with other phenolics of Citrus.

STRUCTURE ET ACTIVITE BIOLOGIQUE D'UNE NOUVELLE PHYTOALEXINE DES CITRUS : LA XANTHYLETINE.
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RESUME - Une coumarine, la Xanthylétine, a été identifiée dans les tissus de Citrus infectés par des *Phytophthora* spp. Cette substance est déterminée par son spectre dans l'Infra Rouge, par Spectrométrie de masse et par résonance magnétique nucléaire. Cette phytoalexine manifeste *in vitro* une importante toxicité pour le *Phytophthora citrophthora* et une synergie avec les autres composés phénoliques des Citrus.

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 ; BAILEY, 1982). The Citrus response to gummosis disease caused by *Phytophthora* spp. ranges from susceptibility to resistance (BOCCAS and LAVILLE, 1978 ; de VALLAVIEILLE, 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.

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MATERIAL AND METHODS

Citrus seedlings, Rough Lemon tolerant and Valencia Late susceptible to gummosis (de VALLAVIELLE 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 (I : I). Further purification was achieved by LH-20 gel column chromatography eluted

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 Mh 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 cladosporioides* inhibition (TAQUET, 1985) and in concavity slides for *P. citrophthora* (RAVISE and KIRKIACHARIAN, 1976) using synthetic medium. The influence of concentration between 25 μg to 200 μ g/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 $CdCl_3$ 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 annelated 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 formule of C14 H12 O3 (Figure 2). Comparison of above data with known physical constants of xanthyletin (STECK, 1971) definitely confirmed the structure determination.

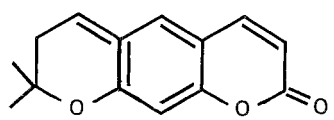

Fig. 2 • STRUCTURE OF XANTHYLETHIN ($C_{14}H_{12}O_3$).

Toxicity test.

The growth of *Cladosporium cladosporioïdes* was not inhibited with 100 μ g 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 25 μg/ml and the growth was completely inhibited with 75 μg/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-

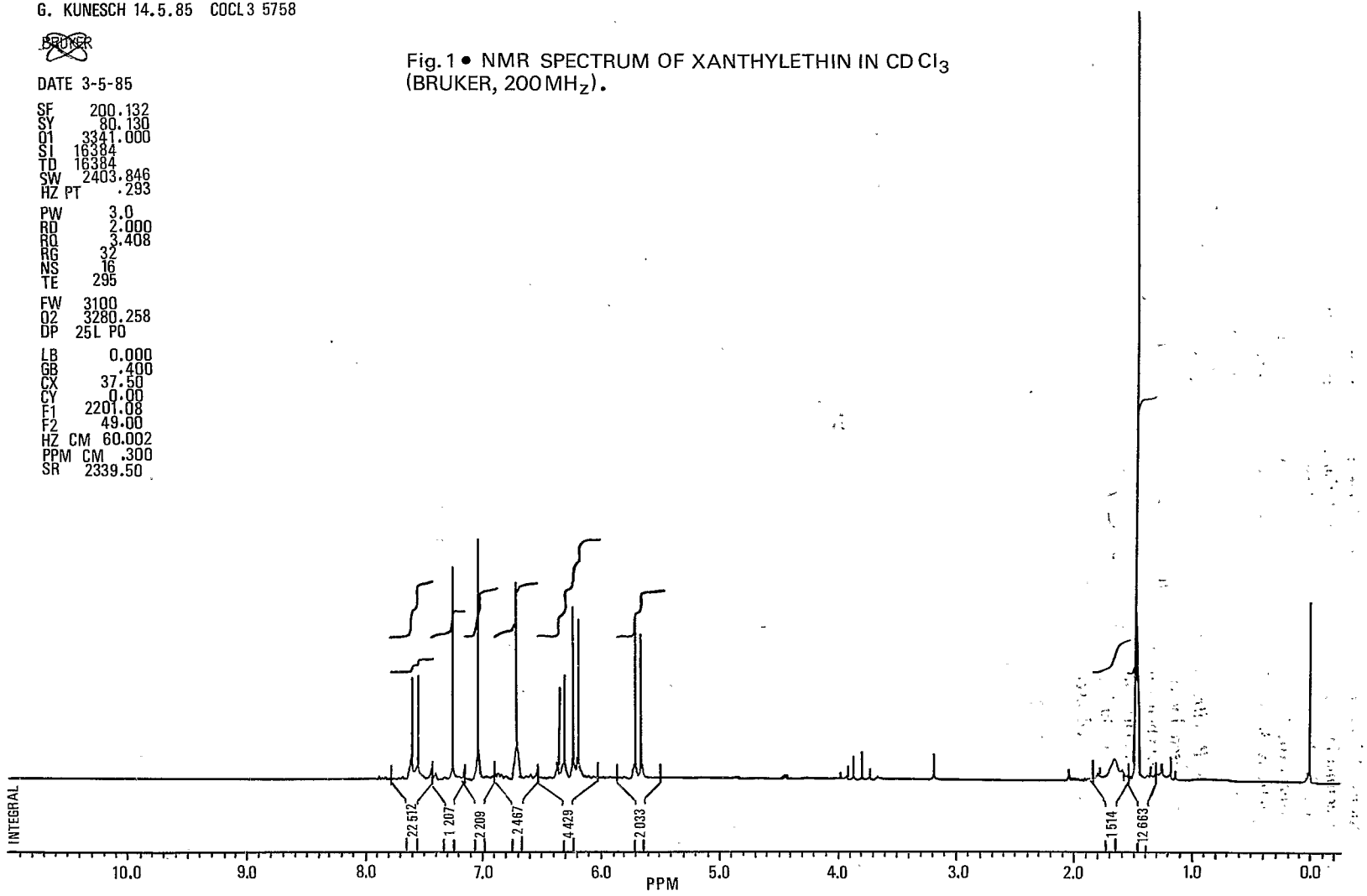

Fig. 1 • NMR SPECTRUM OF XANTHYLETHIN IN $CDCl_3$ (BRUKER, 200 MH_z).

rent concentrations of inhibitor is summarised in table 1. The toxicity of coumarin at 50 μ g/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 occured even in distilled water. According to data of TLC and U.V. spectra, we supposed that an oxidation occured : 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 appearence of nex peaks with λ min. = 275 nm, λmax. 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).

Concentration (μg/ml)	Incubation period				
	24 h	48 h	72 h	96 h	120 h
0	452	762	930	1 156	1 433
25	102	526	690	829	1 160
50	52	217	395	582	904
75	0	0	0	0	0

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.

Substances	Incubation period		
	24 h	48 h	72 h
Xanthyletin	150	580	730
3-4 dimethoxy-benzaldehyde	156	350	535
Xanthyletin + 3-4-dimethoxy-benzaldehyde	0	0	0

DISCUSSION - CONCLUSION

According to previous studies the defence reactions of Citrus are correlated with an increase of synthesis of phenolic compounds, particularly of xanthoxylin (HARTMANN and NIENHAUS, 1974). In our experiment, we have observed an accumulation of this product among the aromatic compounds - benzoic and cinnamic acid derivatives - and of the coumarin after inoculation by *Phytophthora* spp.

Xanthyletin is described as a constituent of *Brosimum rubescens* which is a Rutaceae (GOTTLIEB, 1972). This coumarin is a fungal inhibitor in Citrus similar to pterocarpans which occur in several species of Leguminosae and are synthetized as phytoalexins by Papillonaceae.

The lethal concentration for *P. citrophthora* is greater than the effective dose of other coumarins (in particular 2 - and 3 - methoxy - coumarins) for a strain of *Phytophthora parasitica* (RAVISE and KIRKIACHARIAN, 1976). Xanthyletin is slowly oxidised in aqueous solution and we presume that it is subjected to fungal degradation ; *in vitro* the decrease of toxicity occurs at the same rate as the formation of two metabolites.

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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