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Elicitation of *Platanus* × *acerifolia* cell-suspension cultures induces the synthesis of xanthoarnol, a dihydrofuranocoumarin phytoalexin

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Abstract

The treatment of cell-suspension cultures of *Platanus* × *acerifolia* with the GP66 elicitor produced by *Ceratocystis fimbriata* f. sp *platani* germlings triggers the fast and intense synthesis of a secondary metabolite whose ^{1}H NMR and ^{13}C NMR analysis led to the identification of xanthoarnol. This dihydrofuranocoumarin is principally accumulated in the growth medium reaching the maximum level 48 h after elicitor addition. Xanthoarnol was the major compound produced by elicited cells and showed an inhibitory effect on the germination of conidia of the parasitic fungus. Xanthoarnol is a new phytoalexin described in a plant–fungus interaction. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Platanus* × *acerifolia*; Platanaceae; Plane tree; *Ceratocystis fimbriata* f. sp *platani*; Elicitation; Phytoalexin; Xanthoarnol; Dihydrofuranocoumarin

1. Introduction

The interaction of the London plane tree (*Platanus* × *acerifolia* (Ait) Willd) with the fungus *Ceratocystis fimbriata* f. sp *platani* (Ell and Halst) Walter (*Cfp*) the canker stain agent, has recently received considerable attention since this disease is the most dangerous for the perenity of the plane tree in the French Midi and in other meridional countries. Today, the disease is well known for mechanisms of plant infection (Walter, 1946; Vigouroux, 1979; Bolay & Mauri, 1988), fungus dissemination (Vigouroux, 1987; Vigouroux & Stojadinovic, 1990; Grosclaude, Olivier, Pizzuto, & Romiti, 1991) prophylactic and chemical treatments (Blancard & Vigouroux, 1985; Gessler & Mauri, 1987) to the detriment of plane tree defense responses to the parasitic fungus. However, the synthesis of two coumarin phytoalexins, scopoletin and umbelliferone, has been shown as a major component of defense reactions of young plane tree plants (El Modafar, Clérivet, Fleuriet, & Macheix, 1993). Furthermore, some other neoformed phenolic compounds were also synthetized in response to fungal ingress but their levels were always low and in consequence, their chemical identification was difficult (El Modafar, Fleuriet, Vigouroux, & Clérivet, 1991). Also, for an easier study of phenolic metabolism modifications related to *Cfp* infection, we have used the elicitation of a cell-suspension cultures of *Platanus acerifolia* by a 66 kDa glycoprotein (GP66), the major eliciting compound previously isolated from *Cfp* germlings (Alami, Mari, & Clérivet, 1998). We have checked that scopoletin and umbelliferone and other unknown phenolic compounds effectively accumulate in elicited cell-suspension culture and showed that all these compounds principally accumulate in cell growth medium (Alami et al., 1998). Thus, the extraction and the purification of excreted phenolic

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compounds from growth medium of elicited cells may be an appropriate way to identify these neo-synthetized secondary metabolites. In the present paper, we describe the occurrence and the accumulation in the growth medium of elicited cells of a major compound identified as xanthoarnol, a dihydrofuranocoumarin previously found in *Xanthoxylum arnottianum*. In our case, xanthoarnol is not present before elicitation and it appears to play a role in the regulation of fungus development.

2. Results and discussion

In a previous paper we have reported that the elicitation of plane tree cell-suspension culture with the GP66 elicitor from *Cfp* induced the synthesis and the accumulation of two coumarin phytoalexins, scopoletin and umbelliferone (Alami et al., 1998). Simultaneously, another new compound with purple–blue fluorescence under UV examination of silica gel prep. TLC plates was also synthetized in elicited cells. HPLC analysis of this compound after elution from prep. TLC plates of cell and growth medium extracts revealed an R_t of 28 min and a UV spectrum typical for a coumarin (data not shown). The MS, m/z 262 and the ^{1}H NMR spectrum were in agreement with the structure of xanthoarnol, the natural dihydrofuranocoumarin isolated from the heartwood of roots of healthy *Xanthoxylum arnottianum* Maxim (Ishi, Ishikawa, Sekiguchi, & Hosoya, 1973), a plant of the Rutaceae family. So, ^{1}H NMR (250 MHz, DMSO-d_6) signals were at δ 6.20 (H-3, d, $J=9.8$ Hz), 7.95 (H-4, d, $J=9.8$ Hz), 7.60 (H-5, s), 5.22 (H-3′, d, $J=3.4$ Hz), 4.21 (H-2′, d, $J=3.4$ Hz), 6.81 (H-8, s) and 1.10 (H-5″, d, $J=10$ Hz). Furthermore, ^{13}C NMR (250 MHz, DMSO-d_6) signals were at δ 160 (C-2), 111 (C-3), 144 (C-4), 125 (C-5), 130 (C-6), 160 (C-7), 97 (C-8), 156 (C-9), 98 (C-2′), 69 (C-3′) 70 (C-4″), 27 (C-5″), 26 (C-6″) confirming the identification of the accumulated phenolic compound as xanthoarnol.

Synthesis of xanthoarnol in elicited plane tree cells was fast and intense and it was principally accumulated in growth medium (Fig. 1) as the two others coumarins, scopoletin and umbelliferone (Alami et al., 1998). Xanthoarnol was detected in culture medium from 6 h after elicitor addition at a level of 1.09 μmol g^{-1} FW, then it increased rapidly up to 48 h leading to a high level content, namely 8.8 μmol g^{-1} FW. A very slow decrease was then observed. In cells, the level of xanthoarnol accumulation was very low in comparison to cell medium (Fig. 1). Xanthoarnol was mainly excreted outside the cells 6 and 48 h after elicitor addition, the cell content in xanthoarnol was, respectively, 3.5- and 11.5-fold lower than in growth medium (Fig. 1).

In unelicited control cells, xanthoarnol was only detected as a trace amount after 48 h, namely lower than 0.15 μmol g^{-1} FW in cell growth medium and 0.015 μmol g^{-1} FW in cell extracts (Fig. 1).

Xanthoarnol was the major new phenolic compound synthetized by elicited plane tree cells and accumulated in their growth medium. Although scopoletin and umbelliferone were also principally excreted in elicited cell growth medium, their accumulation levels were significantly lower, 1.35 and 1.6 nmol g^{-1} FW, respectively, as previously measured 48 h after elicitor addition (Alami et al., 1998).

In plant–microorganism interactions, the synthesis of new secondary metabolites triggered during microorganism ingress or treatment with elicitors is generally considered as an active plant defense mechanism (Benhamou, 1996). In *Platanus acerifolia–Ceratocystis fimbriata* f. sp *platani* interaction, such a role for the two coumarins, scopoletin and umbelliferone, has been clearly suggested in leaves of both susceptible and re-

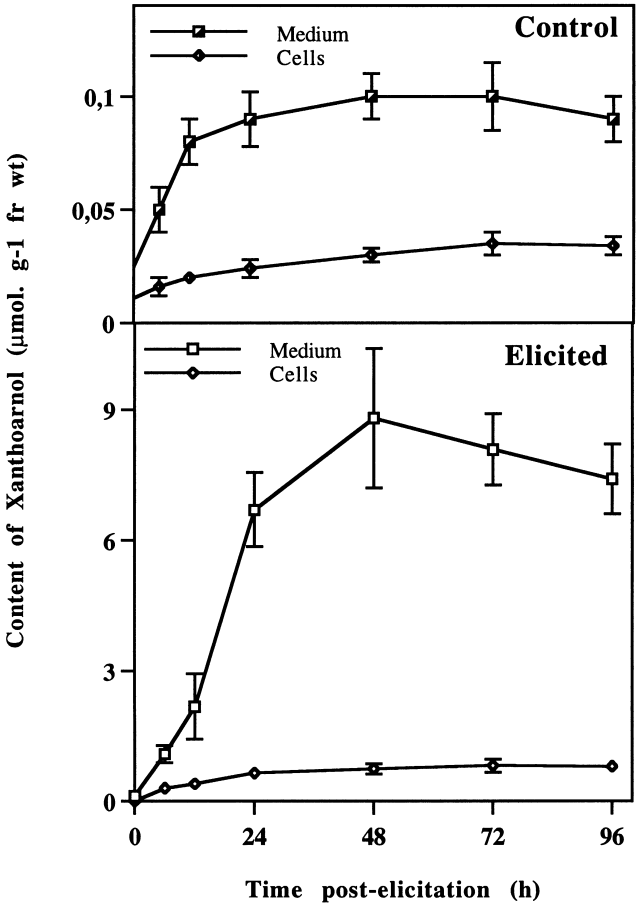

Fig. 1. Time course of xanthoarnol accumulation in cells and growth medium of *P. acerifolia* cell suspension cultures. Cells were treated with the GP66 elicitor (0.5 μg protein equivalent per ml culture medium) from *C. fimbriata* f.sp *platani* germlings (elicited) or with phosphate buffer (control) at day 12 of cell-culture.

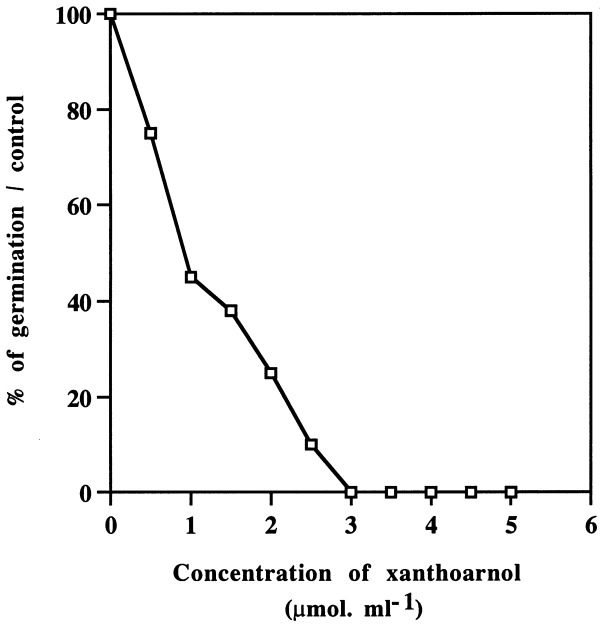

Fig. 2. Effect of xanthoarnol concentration on the germination of *C. fimbriata* f.sp *platani* conidia (48-h-old incubation).

sistant trees and in stems of young plane tree plants (El Modafar et al., 1993; El Modafar, Clérivet, Vigouroux, & Macheix, 1995). For xanthoarnol, in vitro bioassays showed that the ID_{50} for germ tube formation of *Cfp* conidia was approximately 0.85 μM ml^{-1} (Fig. 2) namely to a level near those still established for scopoletin and umbelliferone (0.6 μM ml^{-1}) (El Modafar, Clérivet, Fleuriet, & Macheix, 1993). So, the dihydrofuranocoumarin xanthoarnol may be considered as a new phytoalexin identified in a plant–fungus interaction. Among many known dihydrofuranocoumarins, only (*S*)-marmesin of elicited parsley cells and *Ammi majus* L. cells and (*R*)-ammirin in *A. majus* cells were reported as phytoalexins (Tietjen, Hunkler, & Matern, 1983; Hamerski, Beier, Kneusel, Matern, & Himmelspach, 1990).

It is well established that scopoletin and umbelliferone were accumulated in stem and foliar tissues of plane tree in relation to fungus penetration (El Modafar et al., 1993; El Modafar et al., 1995) but also in reaction to treatment of cell-culture with the GP66 elicitor released from *Cfp* germlings (Alami et al., 1998). In contrast, after experimental inoculation of young plane tree stems, xanthoarnol seems to be present at only very low levels in infected tissues (data not shown) while it is the most accumulated compound in elicited cell-suspension culture. So, whereas the synthesis of the three phytoalexins, xanthoarnol, scopoletin and umbelliferone, was a common response of the two biological systems (whole plant and cell-suspension) to infection or elicitor treatment, the response of cell-suspension culture was strongly amplified for xanthoarnol synthesis. In the whole plane tree plant, the potential role of xanthoarnol in defense against *Cfp* must be really questioned when the content in this antimicrobial compound seems to be low. Indeed, we only know the global content of infected stem fragments but not that of the few cells of the inoculation site involved in plant defense. So, it should be now interesting to study, in the vicinity of inoculation site of plane tree stems, the time course and the level of xanthoarnol accumulation and the activity of some enzymes as prenyltransferases involved in the synthesis of dihydrofuranocoumarin from umbelliferone and demethylsuberosin (Hamerski, Schmitt, & Matern, 1990). We may assume that in addition to the other phytoalexins, scopoletin and umbelliferone, xanthoarnol plays a role in plant defense against the canker stain agent. Finally, the treatment of *P. acerifolia* cell-suspension culture with the GP66 glycoprotein elicitor from *Cfp* may be used as an appropriate experimental model for the elucidation of the regulation of de novo expression of genes involved in plane tree defense responses, particularly through the new expression of dihydrofuranocoumarin metabolism.

3. Experimental

3.1. Cell culture

Cell cultures were generated from calli of *Platanus* × *acerifolia* as previously described (Alami et al., 1998). They were regularly subcultured every 12 days by transferring 10 g of cells (fr. wt) to sterile WPM medium (Russel & McCown, 1986) with 0.15 μM of 2.4-D and kinetin. Cells were grown at 25°C in continuous light (40 μE m^{-2} s^{-1}) under horizontal rotary shaking (95 rpm).

3.2. Elicitation of cell cultures

The GP66 elicitor prepared from a culture filtrate of *Cfp* (Alami et al., 1998) was added to cell culture at day 12 to give a final concentration in the medium of 0.5 μg ml^{-1} protein equivalents. Control (unelicited) cell cultures received only sterile phosphate buffer (10 mM K-Pi buffer pH 7) used for elicitor preparation (Alami et al., 1998). Afterwards, cell cultures were placed under culture conditions as described above.

3.3. Xanthoarnol extraction and quantification

At different points in time (6, 12, 24, 48, 72 and 96 h) following elicitation, 15 ml of cell suspension culture were harvested. The growth medium was collected by vacuum filtration and fresh cells (3 g) were frozen in liquid nitrogen. Growth medium was extracted ×3

with EtOAc (v:v) containing 20% $(NH_4)_2SO_4$ (w/v) and 2% metaphosphoric acid (w/v). The organic phase was reduced to dryness in vacuo and the residue was dissolved in 1.5 ml MeOH. Frozen cells (3 g) were ground in liquid nitrogen then the powder was mixed in a cold EtOH–H_2O (4:1) containing 0.5% Na_2SO_3 (w/v). The extract was then homogenized for 30 s by ultraturax, agitated for 20 min at 4°C and then vacuum filtrated. At the end of EtOH evaporation, the aq. phase obtained was extracted ×3 with EtOAc (v/v) containing 20% $(NH_4)_2SO_4$ (w/v) and 2% metaphosphoric acid (w/v). After dry evapn, the residue was resuspended in 1.5 ml MeOH.

HPLC analysis of cell and growth medium extracts (Waters 990 with a photodiode bar detector coupled with a Waters 420 fluorescence detector) were performed on a spherisorb C18 column (particle size 5 μm, 250 × 5 mm) and xanthoarnol was eluted at R_t 28 min in a solvent consisting of MeCN and H_2O (pH 2.6) using a gradient of 5–18% MeCN for 40 min with 1 ml min^{-1} flow rate. The content in xanthoarnol of cell and growth medium extracts was expressed in μmol of xanthoarnol g^{-1} fr. wt (FW). The MeOH extracts from cells and growth medium were also applied to silica gel prep.TLC plates with fluorescence indicator and were chromatographed with $CHCl_3$–HOAc–H_2O (4:1:1). A purple–blue fluorescent compound with R_f 0.27 was detected in the extracts by examination under UV light (254 and 366 nm) and appropriate bands were scraped off, eluted with $CHCl_3$–MeOH (1:1) and analysed by HPLC as described above for cells and growth medium methanolic extracts. The data correspond to 3 replicates with 3 flask samples.

3.4. Xanthoarnol ^{1}H NMR and ^{13}C NMR analysis

^{1}H NMR and ^{13}C NMR were performed on a Bruker AC-250 spectrometer operating at 250 MHz in DMSO-d_6 and with TMS as int. standard.

3.5. Mass spectrometry of xanthoarnol

Mass spectrum was obtained on a Hewlett-Packard 5989A mass spectrometer (70 eV) coupled with an HPLC system equipped with a spherisorb ODS-2 C18 column (particle size 5 μm, 250 × 4 mm). Xanthoarnol was eluted in a solvent consisting of MeCN and bidistilled water using a gradient of 40–85% MeCN for 10 min then decreasing to 40% up to 13 min with 1 ml min^{-1} flow rate.

3.6. Bioassays

A *Cfp* conidia suspension in sterile distilled water (4×10^4 spores ml^{-1}) was prepared from a thallus cultivated 24 h on potato dextrose agar medium, then it was placed in contact with xanthoarnol solutions in DMSO sterilized by filtration on a 0.2 μm membrane. The final DMSO concentration, identical in test and control, was 2.5%. Xanthoarnol concentrations were 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 μM ml^{-1}. Incubation occurred at 25°C in the dark. After 48 h, the rate of germination was estimated by microscopic examination of conidia (10 samples of 100 conidia).

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References

Alami, I., Mari, S., & Clérivet, A. (1998). *Phytochemistry*, *48*, 771.
Benhamou, N. (1996). *Trends Plant Sci.*, *1*, 233.
Blancard, D., & Vigouroux, A. (1985). *Phytoma*, *343*, 51.
Bolay, A., & Mauri, G. (1988). *Rev. Hortic. Suisse*, *61*, 77.
El Modafar, C., Fleuriet, A., Vigouroux, A., & Clérivet, A. (1991). *Ann. ANPP*, *1*, 149.
El Modafar, C., Clérivet, A., Fleuriet, A., & Macheix, J. J. (1993). *Phytochemistry*, *34*, 1272.
El Modafar, C., Clérivet, A., Vigouroux, A., & Macheix, J. J. (1995). *Eur. J. Plant. Pathol.*, *101*, 503.
Gessler, C., & Mauri, G. (1987). *Bot. Helv.*, *97*, 349.
Grosclaude, C., Olivier, R., Pizzuto, J. C., & Romiti, C. P. M. H. (1991). *Rev. Hortic.*, *317*, 42.
Hamerski, D., Beier, R. C., Kneusel, R. E., Matern, U., & Himmelspach, K. (1990). *Phytochemistry*, *29*, 1142.
Hamerski, D., Schmitt, D., & Matern, U. (1990). *Phytochemistry*, *29*, 1131.
Ishi, H., Ishikawa, T., Sekiguchi, H., & Hosoya, K. (1973). *Chem. Pharm. Bull.*, *21*, 2346.
Russel, J. A., & McCown, B. H. (1986). *Plant Science*, *46*, 133.
Tietjen, K. G., Hunkler, D., & Matern, U. (1983). *Eur. J. Biochem.*, *131*, 401.
Vigouroux, A. (1979). *Eur. J. For. Path.*, *9*, 31.
Vigouroux, A. (1987). *Prog. Agric. Vitic.*, *104*, 529.
Vigouroux, A., & Stojadinovic, B. (1990). *Eur. J. For. Path.*, *20*, 118.
Walter, J. M. (1946). *US Dept. Agric. Circ.*, 742.