Xanthotoxin and Other Furanocoumarins as Phytoalexins in *Pastinaca sativa* L. roots

Fatma M. Al-Barwani and Elsadig A. Eltayeb

Department of Biology, College of Science, Sultan Qaboos University, P.O. Box 36, Al Khod 123, Muscat, Sultanate of Oman, Email: eatayeb@squ.edu.om.

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ABSTRACT: Our results confirm the presence of the furanocoumarins psoralen, xanthotoxin, isopimpinellin, angelicin and bergapten as normal constituents of *Pastinaca sativa* roots. However, the amounts of these compounds were increased in induced roots. The amount of increase in the induced roots varied depending on the inducing agent and time of incubation. In addition the results show for the first time the presence of pimpinellin and isobergapten as constitutive antifungal compounds in *P. sativa* roots.

KEYWORDS: *Pastinaca sativa,* angelicin, psoralen, isopimpinellin, bergapten, pimpinellin, isobergapten, antifungal compounds.

1. Introduction

Many workers have reported elicitation of phytoalexins by various biotic factors. The most widely used and natural way for the induction of phytoalexin production is by fungal infection. In addition to biotic elicitors, salts of heavy metals and UV-light have commonly been used to elicit phytoalexins in many plants.

Different elicitors vary in their ability to induce phytoalexins depending on the plants induced and the phytoalexin produced. For example, ultraviolet radiation was found by Mercier *et al.* (1993) to be the only

elicitor effective in eliciting the accumulation of the carrot phytoalexin, 6-methoxymellein (**1**) in carrot root slices at cold storage temperatures, while Edwards and Strange (1991) in their test of the ability of various biotic and abiotic agents, found that UV light and heavy metals only resulted in low levels of accumulation in groundnut leaflets. On the other hand, wounding by detaching leaflets from plants and inoculation of groundnut (*Arachis hypogaea* L.) leaflets with non-pathogenic fungi as well as naturally infected plants with major fungal pathogens resulted in the elicitation of phytoalexin response in groundnut with variation in the yield according to the challenging organism (Subba Rao and Strange 1994).

Parsnips are known to contain at least six furanocoumarins namely, xanthotoxin (**2**), bergapten (**3**), isopimpinellin (**4**), angelicin (**5**), psoralen (**6**) and (**7**) imperatorin (Johnson *et al*., 1973; Berenbaum *et al.,* 1984; Desjardins *et al*., 1989; Ekiert and Gomólka, 2000). Johnson and co-workers (1973), however, have established that xanthotoxin is a phytoalexin in parsnip roots by demonstrating its 20-fold induction in the roots inoculated with several fungi nonpathogenic to parsnip.

In this study, phytoalexin induction in *P. sativa* was investigated using fungi and the abiotic elicitor $CuCl_2$. Quantitative analysis using HPLC was used to compare the effectiveness of these elicitors in inducing phytoalexins. Time-course accumulation of phytoalexins was also followed by HPLC.

2.   Materials and Methods

2.1   Plant material and Fungi

Parsnip roots purchased from a local market (cultivar unknown) were surface sterilized in 1% sodium hypochlorite, and then rinsed twice with distilled water before use.

Dreschslera sorkiniana Sacc. *Fusariu*m spp. and *Alternaria* spp. were obtained from the College of Agriculture, Sultan Qaboos University, Muscat, Oman.

Fungi were grown on Petri dishes containing potato-dextrose agar medium and were incubated at 24 °C. Spore suspensions were obtained by flooding cultures that produced spores or conidia with deionized water containing drops of Tween-20 and gently scraping the surface. Mycelial fragments were removed by passing the spore suspensions through layers of lens tissue and the density was adjusted to approximately 1.0×10^6 spores/ml with the aid of a haemocytometer.

2.2   Chemicals

The following standards were used for TLC and HPLC analysis: isopimpinellin from Carl Roth (Karlsruhe, Germany); psoralen, xanthotoxin and bergapten from Aldrich (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and angelicin from Sigma (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Only HPLC grade solvents were used for HPLC analysis.
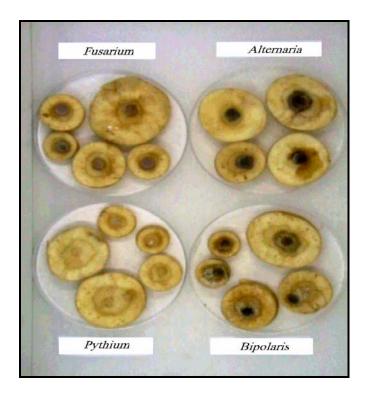
2.3   Induction of Phytoalexins
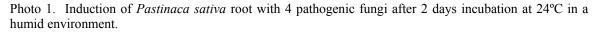
2.3.1 Chemical Induction

Parsnip roots were chemically induced with a 50µl aliquot of 10mM $CuCl_2$ containing drops of Tween-20, placed on the fresh slices of sterilized root in sterile Petri dishes lined with moistened filter paper. The Petri dishes were then sealed with Para film and incubated in a humid environment at 24 ºC. The control slices received water containing drops of Tween-20 but were otherwise treated in the same way. After 48 hr incubation, the slices were harvested for the determination of phytoalexin production.

2.3.2 Biotic Induction

Biotic induction was achieved by placing freshly sliced parsnip root disks on Petri dishes containing a moistened filter paper. An agar inoculum plug (5mm diameter), mycelial side down, was then placed in the center core of each disk and incubated as above. Inoculum plugs were cut from the growing margins of not more than 10 day-old cultures of a fungus. Control slices received agar plugs without fungus (photo 1).

Photo 1. Induction of *Pastinaca sativa* root with 4 pathogenic fungi after 2 days incubation at 24ºC in a humid environment.

2.4   Extraction and Detection of Phytoalexins

Induced and control parsnip slices were extracted twice overnight with methanol. The dried extracts were then dissolved in 100% methanol for phytoalexin analysis.

A thin layer chromatography (TLC)-*Cladosporium* bioassay was used for the detection and screening of phytoalexins. About 20 µl aliquot of the concentrated extracts were applied to TLC plates (Si gel 60 f-254, 20 X 20 cm). The plates were developed in either chloroform-methanol (97:3), or in toluene-ethyl acetate (1:1) and air dried. The developed TLC plates were sprayed with a dense spore suspension of *Dreschslera sorkiniana* in a nutrient solution. The plates were then incubated at 24 °C in a moist chamber for 2-5 days. The antifungal inhibition zones were observed and the R_f values calculated.

The spore suspension nutrient solution was prepared by mixing 20 ml salt solution and 10 ml of 15 % glucose solution. 1 L of salt solution contained 7g KH_2PO_4, 4g KNO_3, 3g $NaHPO_4.2H_2O$, 1g $MgSO_4.7H_2O$.

2.5   Identification and Quantification of Phytoalexins

After the TLC bioassay, using the above mentioned solvent systems, the regions of thin layer chromatograms corresponding to the inhibitory zones were scraped off and eluted with 100% methanol. Identification of the antifungal compounds was carried out by Co-TLC and Co-HPLC with authentic standards or by comparison of R_f, R_t values and UV spectra with literature.
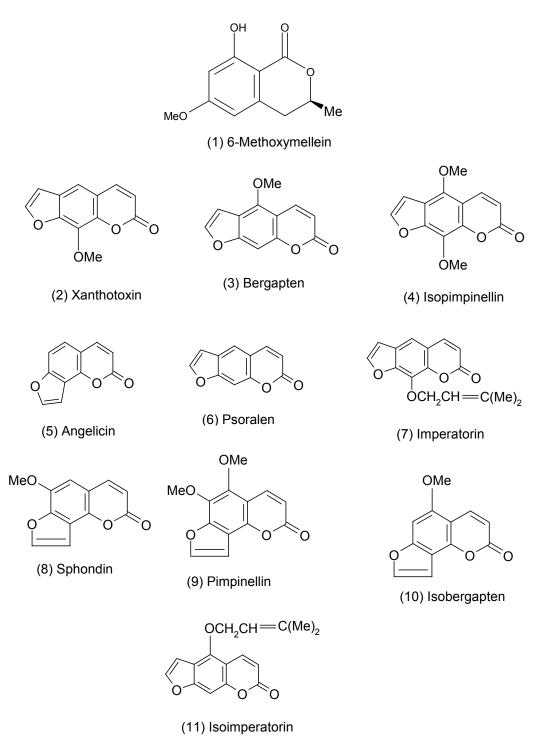
(1) 6-Methoxymellein

(2) Xanthotoxin

(3) Bergapten

(4) Isopimpinellin

(5) Angelicin

(6) Psoralen

(7) Imperatorin

OCH$_2$CH$=$C(Me)$_2$

(8) Sphondin

(9) Pimpinellin

(10) Isobergapten

OCH$_2$CH$=$C(Me)$_2$

(11) Isoimperatorin

Figure 1. Chemical structures of some phytoalexins

2.5.1   Thin Layer Chromatography

Purification of the eluted antifungal compounds was carried out on TLC plates (Si gel 60 f-254, 20 X 20 cm). The chromatograms were developed at room temperature using the following solvent systems:
A)   Chloroform: Ethyl acetate (2:1).
B)   Chloroform.
C)   Toluene: Ethyl acetate (1:1)

Following evaporation of the solvent, the thin layer plates were observed under UV light and the R_f values were noted. Compounds were identified from comparison of their R_f values and fluorescence under UV light (254 nm) with standards run parallel to the samples.

2.5.2   HPLC analysis

A Waters gradient system consisting of Waters 626 pump, a Model 600s system controller, a Waters 717 plus auto-sampler and a Waters in-line degasser was used for the separation and identification of antifungal compounds from the crude extracts or from the eluted TLC bands. For UV detection a Waters 996 photo-diode array detector was used at 254 nm. The gradient separations were carried out using a Waters Spherisorb ODS-II (3 μm) cartridge column (100 x 4.5 mm I.D.). Solvent A was tetrahydrofuran-H_2O (3.5:96.5) and solvent B was 100% MeOH. The gradient profile was as follows: 0-20 min, 25% B in A (isocratic); 20-40 min, 25-45% B in A (linear gradient); 45-55 min, 45% B in A (isocratic). All standards and samples were dissolved in MeOH and the injection volume was 10μl. Peaks of sample and standard compounds were identified and compared by on-line UV detection at 254 nm.

2.6   Antifungal Activity

2.6.1 Slide Bioassay

Samples 25 μl of a series of concentration of the antifungal compounds dissolved in methanol were placed on a glass microscope slide within a circle of vaseline. These were left to dry before 50 μl of spore suspension was added. Slides were incubated in a plastic container lined with moist tissue paper at 25 °C in the dark for 16 to 24 hr. Spore germination and germ tube elongation were determined by microscopic observation.

2.6.2 Paper-disk Method

Paper discs (0.3cm diam.) were loaded with a known amount of the antifungal compound in absolute methanol (maximum volume, 20 μl). Control disks were treated with 20 μl of methanol alone. In all cases, sufficient time was allowed for the methanol to evaporate before transferring the discs to agar surfaces. Discs were arranged in Potato Dextrose Agar (PDA) Petri plates, which had been inoculated with the test fungi. Plates were incubated at 25 °C and diameters of inhibiting zones were measured after 20 hr. Percentage inhibition was calculated as follows:

100- {mycelial growth in treated/ mycelial growth in control × 100}

EC_{50} values were calculated from the regression lines obtained from plots of concentration versus percent inhibition.

3.   Results

3.1 Chemical Induction

Strong antifungal zones on TLC were produced in both fungal and copper chloride treated parsnip root slices that were also present in the control slices. These compounds were identified by on-line UV detection (Figure 2) and found to be the previously reported furanocoumarins xanthotoxin, isopimpinellin, angelicin and bergapten (Desjardins *et al.*, 1989; Johnson *et al*., 1973; Ekiert and Gomólka, 2000). Another

previously reported compound, sphondin (**8**) (R_t 30) and two compounds which have not been reported from parsnip roots before, pimpinellin (**9**) (R_t 41) and isobergapetn (**10**), (R_t 43), were identified by their properties under UV light and by comparing their retention times and UV spectra with literature values (Erdelmeier *et al.*, 1985). However, isoimperatorin (**11**), earlier reported to be a constitutive component of parsnip roots was not detected in any of our samples, but another unidentified compound was detected at R_t 37. Surprisingly, psoralen was detected only in the control samples at very low concentration and was absent from the treated samples.
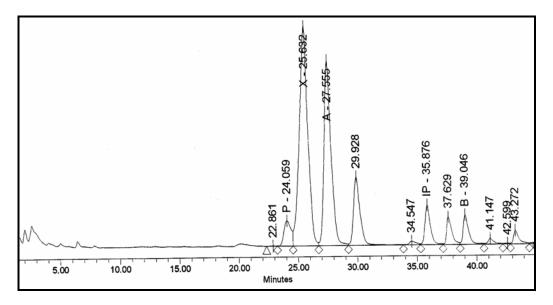

Figure 2. Gradient HPLC separation of furanocoumarins from *Pastinaca sativa* root. Separations were carried out using a Waters Spherisorb ODS-II (3 μm) cartridge column (100 x 4.5 mm I.D.). Solvent A was tetrahydrofuran-H_2O (3.5:96.5) and solvent B was 100% MeOH. P: psoralen; X: xanthotoxin; A: angelicin; B: bergapten; IP: isopimpinellin. R_t 29.9 Sphondin; Rt 43.2: isobergapten; R_t 41.1: pimpinellin.

Quantitative HPLC analysis indicated that the amounts of all of these compounds increased in the induced slices but at different levels (Figure 3). The largest increase is in angelicin which increased by 34 fold in the chemically induced slices from 0.015 mg/g fresh weight in the control to 0.51 mg/g fresh weight followed by xanthotoxin which increased by 29 fold in the chemically induced slices from 0.05 mg/g fresh weight in the control to 1.48 mg/g fresh weight (Table 1).

Table 1. Increased furanocoumarin concentrations in *Pastinaca sativa* L. root after 1 day induction with $CuCl_2$.

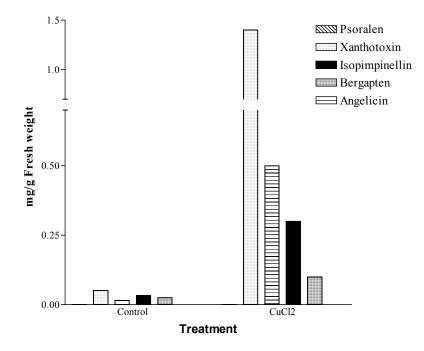| Coumarin	mg/ g of Fresh weight		Increase Test/Control
	Control	Test	
Psoralen	0.001	n.d.*	-
Xanthotoxin	0.051	1.475	29
Isopimpinellin	0.033	0.392	12
Bergapten	0.025	0.172	7
Angelicin	0.015	0.51	34

* Not determined

12

Figure 3. Furanocoumarin content in Pastinaca sativa L. root after one day incubation with $CuCl_2$.

3.2 Biotic Induction

Parsnip roots inoculated with four different fungi resulted in increased levels of the furanocoumarins psoralen, xanthotoxin, isopimpinellin, bergapten and angelicin (Figure 4). The five furanocoumarins were present in small amounts in control parsnip roots. Both the amounts and proportions of the furanocoumarins varied with the type of elicitor used. The largest increase in furanocoumarin content was observed in parsnip roots inoculated with the fungus *B. sorkiniana,* while the smallest increase was in parsnip roots inoculated with *Alternaria* spp. In all treatments, xanthotoxin was the major phytoalexin induced in parsnip roots and was the major constituent of the control roots.

Xanthotoxin, angelicin and psoralen were the major phytoaleins induced in parsnip roots by the fungus *B. sorkiniana*. Treatment with copper chloride however, resulted in the accumulation of xanthotoxin and isopimpinellin as the major induced compounds, while psoralen was not detected in these discs. In addition, three other furanocoumarins, sphondin, pimpinellin and isobergapten were found in all treatments including the control but the amounts of these compounds were not determined.

3.3 Time-course Accumulation of Phytoalexins

Changes in furanocoumarin content in parsnip root discs after induction with copper chloride solution over a period of six days are shown in Figure 5. Increase in furanocoumarin concentrations was observed from day 1 after induction. At this time, xanthotoxin and angelicin reached their maximum concentrations of 1.48 and 0.51 mg/g fresh weight respectively, followed by a rapid decline at day 2. While isopimpinellin and bergapten continued to increase to their maximum 0.60 and 0.26 mg/g fresh weight, respectively, at day 2. Traces of psoralen were first observed at day 5, which increased to 0.052 mg/g fresh weight at day 6.

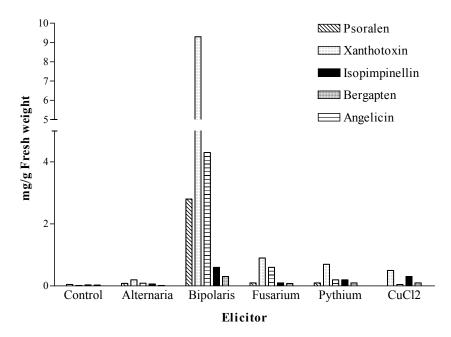Xanthotoxin remained the dominant furanocoumarin throughout the experiments.

13

Figure 4. Furanocoumarin content in *Pastinaca sativa* L. root treated with different elicitors elecicitors after 3 days incubation .
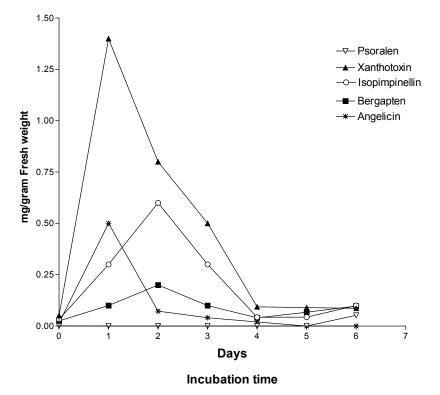

Figure 5. Time course accumulation of furanocoumarin *Phytoalexins sativa* L. roots induced with copper chloride.

14

3.4   Antifungal Activity

Three pathogenic fungi: *Alternaria* spp*. Bipolaris* spp. and *Fusarium* spp. were tested for their sensitivity towards three antifungal compounds detected in *P. sativa*. The fungitoxic activity of the three compounds on the spore germination and the mycelial growth on agar plates are shown in Table 2.

All the tested furanocoumarins had relatively high EC_{50} values on both spore germination and mycelial growth of the tested organisms. The tested compounds inhibited spore germination of *Alternaria* and *Bipolaris* more effectively than mycelial growth of *Alternaria* spp. but were most effective in inhibiting mycelial growth of *Fusarium* spp.

Table 2. Effective concentrations of furanocoumarins resulting in 50% inhibition of conidia germination and growth inhibition for three fungi.

Compound	EC_{50} (µg/ml)			
	Conidia germination		Mycelial growth	
	Alternaria	*Bipolaris*	*Alternaria*	*Fusarium*
Xanthotoxin	1920	4320	5560	195
Bergapten	n.d.[*]	1920	6010	n.d.*
Angelicin	3460	n.d.*	6120	115

* Not determined

The high EC_{50} values of the furanocoumarins obtained here for mycelial growth of *Alternaria* spp. are comparable to the values obtained by Afek *et al*. (1995a and b) for celery.

4.   Discussion

Johnson *et al.* (1973) reported the production of xanthotoxin as a phytoalexin of *Pastinaca sativa* roots inoculated with several fungi nonpathogenic to parsnip. This finding was confirmed in this study but the amounts of xanthotoxin reported here were larger than those reported by Johnson and his co-workers. Induction of parsnip roots with $CuCl_2$ in the present study resulted in an increase in the amounts of xanthotoxin by 29- fold compared to 20- fold.   Although xanthotoxin was found to be the major furanocoumarin component of parsnip root both constitutively (0.051 mg/g fresh weight) and $CuCl_2$ induced (1.47 mg/g fresh weight), the present study shows that xanthotoxin is not the only induced compound in parsnip roots. All other reported constitutive furanocoumarins of parsnip root, with the exception of psoralen and imperatorin, were found to increase similarly in the induced samples. The induced furanocoumarins are bergapten, isopimpinellin, angelicin and sphondin. A large increase in angelicin, psoralen, isopimpinellin and bergapten was also observed in parsnip roots inoculated with *B. sorkiniana*. This is therefore the first confirmation that psoralen, isopimpinellin, bergapten and angelicin are phytoalexins in parsnip roots with a similar effect to xanthotoxin.

The large increase in furanocoumarins as a result of induction is in agreement with the earlier reports on the response of the Umbelliferae to phytoalexin induction. For example, Chaudhary *et al*. (1985) considered the linear furanocoumarins, psoralen (**6**), xanthotoxin and bergapten (**7**) to be phytoalexins of celery (*Apium graveolens*) by reporting that diseased celery infected with the fungus *Sclerotinia sclerotiorum* had greatly increased the levels of these compounds that are normally found in healthy celery (Beier and Oertli, 1983; Chaudhary *et al*., 1985; Ashwood-Smith *et al.*, 1986; Trumble *et al*., 1990). Similarly, Al-Barwani (2002) in her survey of phytoalexins in a number of umbelliferous species found that the majority of the species studied produce high levels of furanocoumarins as a result of induction. In many cases, the induced compounds were also found constitutively in small amounts in the untreated tissue.

15

The use of a gradient HPLC separation of furanocoumarins with on-line UV detection and Spherisorb ODS II (3 μm) column at the described gradient profile (Erdelmeier *et al*., 1985) in this study has allowed the separation and identification of two more furanocoumarins in *Pastinaca sativa* roots. These two compounds, pimpinellin and isobergapten were not quantified; hence their concentrations in the control and induced samples were not compared. Whether they also act as phytoalexins in parsnip requires further investigation.

Variation in the amount and proportion of phytoalexins was observed in our study with *P. sativa* roots following treatment with various biotic and abiotic elicitors. The biotic elicitor, *B. sorkiniana* was found to be the most effective. Parsnip roots induced with other fungi resulted in low levels of furanocoumarin accumulation while the heavy metal copper chloride was found to be less effective.

Time course studies of phytoalexin accumulation in *P. sativa* root showed variation in the rate of accumulation of the different compounds using the same non-specific elicitor, copper chloride. Thus the major phytoalexins, xanthotoxin, and angelicin accumulated at day 1 while isopimpinellin and bergapten accumulated at day 2 after elicitation. This variation in the rate of accumulation of different phytoalexins of the same species was also observed elsewhere, e.g. in *Conium maculatum* L. leaves following elicitation with $CuCl_2$ (Al-Barwani and Eltayeb, 2004). In both species, psoralen was not detected until day 6 or 7 after elicitation. This was accompanied by a decrease in the levels of other coumarins. This is probably due to a decrease in the rate of transformation of psoralen (the presumed biosynthetic precursor of the other furanocoumarins) to other alkoxy-furanocoumarins. This was similarly observed in celery following elicitation with copper sulfate in cold conditions (Beier and Oertli, 1983).

While the concentrations of xanthotoxin and angelicin in the induced roots of *Pastinaca sativa* were found to be lower than their EC_{50} values on mycelial growth of *Alternaria* and conidia germination of both *Alternaria* and *Bipolaris*, the concentrations of the two compounds were well above the concentrations required for 50% inhibition of mycelial growth of *Fusarium*. Thus, despite their high EC_{50} values, these compounds are induced in the Umbelliferae at levels high enough to cause inhibition of both conidia germination and mycelial growth of some pathogenic fungi.

However, it should be noted that the EC_{50} values were compared with the concentrations in the chemically induced tissues. Since variation in the amounts of phytoalexins with the use of different elicitors was observed in the present study, the concentration of these compounds may be greater in infected tissue and thus play a role in disease resistance. To prove the role of these compounds in disease resistance, further studies are required to demonstrate that the substances are present at the right time, in the right place and in sufficient quantity to cause any observed inhibition of microbial colonization of plant tissue (Mansfield, 2000).

5.   Acknowledgment

6.   References

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