

The Production of Pyrenocines A and B by a Novel *Alternaria* species

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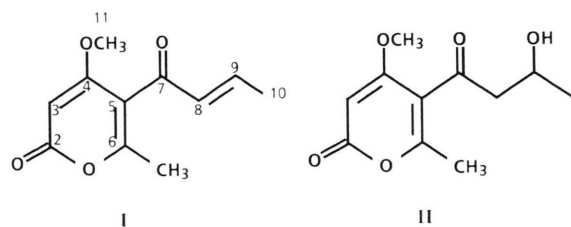
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Pyrenocine A, Phytotoxin, Fungal Metabolite, *Alternaria*, *Helianthus tuberosus*

A previously undescribed species of *Alternaria* was isolated from the leaves of *Helianthus tuberosus* bearing necrotic lesions. When this isolate was grown in liquid medium two phytotoxic metabolites were produced. These compounds were identified as pyrenocine A and pyrenocine B. The former metabolite was found to be lethal to both isolated protoplasts and whole leaf tissue of *Helianthus*.

Introduction

Two metabolites which have been assigned the trivial names pyrenocine A and pyrenocine B, were first described by Sato *et al.* as products of the onion pink root fungus *Pyrenochaeta terrestris* [1]. Subsequently these authors revised the structures of both compounds, following X-ray crystallographic studies, as shown below for pyrenocine A (**I**) and pyrenocine B (**II**) [2]. Pyrenocine A was thus shown to be identical to citreopyrone which was reported by Niwa *et al.* [3] as a metabolite of *Penicillium citreoviride*. Members of the fungal genus *Alternaria* are known to be versatile in their synthesis of phytotoxic secondary metabolites [4–6]. In this report we describe the isolation of a novel *Alternaria* species which produced two toxic compounds which were identified as I and II. The fungus was isolated from diseased leaves of the Jerusalem artichoke (*Helianthus tuberosus* L., Compositae), a plant commonly grown for its edible tubers and as a commercial source of inulin.



Pyrenocine A (**I**) and pyrenocine B (**II**).

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Results and Discussion

The fungus used in this study was isolated from leaves of greenhouse-grown *H. tuberosus* bearing numerous necrotic lesions. This isolate was maintained in pure culture on V-8 juice agar. Following incubation on this medium, at 28 °C under continuous illumination provided by cool white fluorescent lights, sporulation readily occurred. Microscopic observation of sporulating cultures revealed muriform conidial morphology typical of the genus *Alternaria* [7, 8]. However, this species is morphologically and physiologically distinct from the sunflower pathogen *Alternaria helianthi* (Hansf.) Tubaki and Nishihara [9] which is pathogenic to many members of the genus *Helianthus* including *H. tuberosus* [10]. The assignment of the novel isolate as a member of the genus *Alternaria* was subsequently confirmed independently [11]. The organism was grown in liquid shake cultures on either modified Czapek Dox broth (medium CD) [12] or on medium derived from the aqueous extract of the fresh leaves of the sunflower plant (*Helianthus annuus* L.). This latter medium is referred to here as sunflower extract medium (SEM). Incubation of liquid cultures was performed at 28 °C on a gyratory shaker operating at 170 rpm. After growth for 3–4 weeks the culture filtrate was partitioned against diethyl ether. The organic phase was chromatographed on thin layer plates of silica gel using diethyl ether as developing solvent. Compounds I and II were visualised on chromatograms under short wavelength UV light. Further purification of the two metabolites, when necessary, was achieved by HPLC on a reverse phase C₁₈ µBondapak column. Under these conditions I and II eluted with retention times of 3.63 and 2.94 respectively. Their identification was achieved by spectral comparison (UV, MS and ¹H NMR) with authentic samples of pyrenocine A and B isolated from cul-



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tures of *P. terrestris*. Both compounds readily crystallized as colourless needles from chloroform/ethanol. The phytotoxin related metabolite pyrenocine C [13] reported to co-occur with pyrenocines A and B in cultures of *P. terrestris* was not encountered in the present investigation.

In reports on the production of the pyrenocines by the onion pink root pathogen, *P. terrestris*, bioassays for phytotoxicity have examined their effect on the inhibition of seedling elongation in various species [1, 14]. In the present investigation, since the fungus was associated with the aerial parts of the plant, we examined the effect of pyrenocine A on leaves of the commonly cultivated oil seed crop plant, *Helianthus annuus*, as well as the response of *H. tuberosus* to this toxin. In phytotoxicity assays utilising whole leaf tissue of *H. annuus*, pyrenocine A applied as an aqueous solution in 2% dimethyl sulfoxide (DMSO) resulted in a necrotic lesion at concentrations of 12 ppm or lower, within 4 days of application (Table I). Of the two plant species examined *H. annuus* was more sensitive to the toxin over the whole concentration range in each of two treatments, as compared with *H. tuberosus*. Thus the former species exhibited symptoms more rapidly than the latter following similar application of the compound at the same concentration, and subsequent symptom expression was more severe in the case of *H. annuus* than for *H. tuberosus*. For example, 24 h following the application of 3.3 µg pyrenocine A in a 10 µl droplet *H. annuus* showed a dark brown necrotic spot 2 mm in diameter. The corresponding lesion for *H. tuberosus* was a grey-brown necrotic fleck with a diameter of ≤ 1 mm. The assay results indicating differential toxicity to the two *Helianthus* species are

illustrated further in Table I which gives the size of the necrotic lesion 93 h post-application. The induction of a necrotic response following the application of pyrenocine A was manifest both in the presence and absence of a laceration of the leaf cuticle, demonstrating the relatively rare ability of pyrenocine A among toxic fungal metabolites to readily penetrate intact leaf surfaces. However, predictably, symptoms appeared earlier and/or at lower concentrations in the presence (as opposed to the absence) of a needle puncture (Table I). As well as necrosis, yellowing of leaf tissue surrounding areas of application of pyrenocine A was generally observed. Pyrenocine A was also toxic to leaf tissue of the noxious weed *Abutilon theophrasti* Medic., although levels of activity were less than those expressed in *Helianthus* (unpublished results).

Bioassays were also performed with isolated protoplasts of *H. annuus*. In this case activity was monitored by decrease in protoplast viability using the fluorescein diacetate staining technique [15]. After incubation in the presence of different levels of pyrenocine A for 4 days, values for the viability of isolated protoplasts, expressed as a percentage of viability of the control treatment, were recorded as zero and 73% for concentrations of 30 and 0.3 ppm respectively. These concentrations represent the highest and lowest levels tested (Fig.). By extrapolation the LD 50 value of pyrenocine A for sunflower protoplasts is approximately 1 ppm. Thus the *Helianthus* protoplast viability technique is, as expected, a more sensitive bioassay for compound I than those utilising whole leaf tissue of sunflower [14, 16]. Similarly, pyrenocine A was previously demonstrated to express high levels of activity against isolated proto-

Table I. Effect of pyrenocine A on whole leaf tissue of two *Helianthus* spp.^a

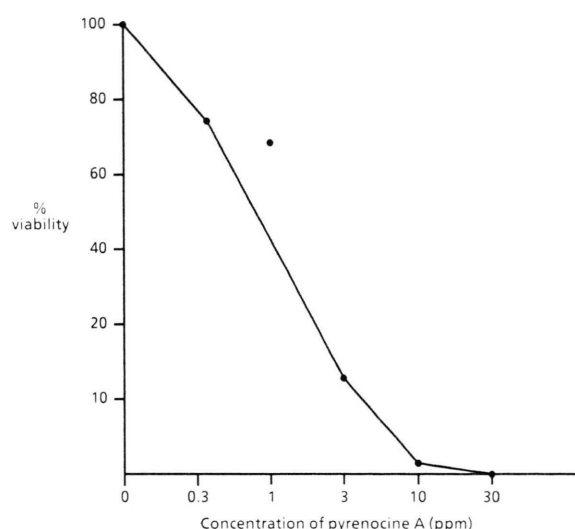
Concentration [ppm]		Diameter of necrotic lesion [mm]						(control)
		330	110	37	12	4	1.4	
<i>H. annuus</i>	Treatment 1 ^b	5	2.5	1.5	1	0.5	0.5	— ^c
<i>H. annuus</i>	Treatment 2	4.5	1	—	—	—	—	—
<i>H. tuberosus</i>	Treatment 1	3.5	2	1	0.5	—	—	—
<i>H. tuberosus</i>	Treatment 2	1	1	—	—	—	—	—

^a Leaves were scored for necrosis to the nearest 0.5 mm 93 h post-application (see text).

^b Treatment 1-droplets were applied over needle punctures on the leaf surface; Treatment 2-droplets were applied to intact leaf surfaces.

^c — = no observed effect.

Fig. Effect of pyrenocine A on the viability of *Helianthus* protoplasts*.



* Viability was scored after treatment for 4 days and is expressed relative to the viability of untreated control protoplasts (64% viability after 4 days).

plasts of onion (*Allium cepa* L., Liliaceae) (LD 50=0.4 ppm) [14]. Our current observations are thus not only consistent with the earlier report on the potent activity of pyrenocine A against protoplasts but also serve to amplify and reiterate the earlier findings [1, 14] on the lack of specificity of this metabolite with respect to taxonomy of the target organism.

Pyrenocine B was not assayed for phytotoxicity in these studies. The apparent weak activity of pyre-

nocine B previously reported against seedling elongation [1] may be explained by its spontaneous conversion in aqueous media to the toxic pyrenocine A, as postulated by Sparace *et al.* [14]. The same authors reported pyrenocine B to lack toxicity to onion protoplasts [14].

Mycelial growth from cultures grown on the complex SEM medium was considerably less than that obtained from modified Czapek-Dox broth, the carbohydrate concentration of which was 20 times higher than that of SEM (Table II). After culture for 21 days on SEM, concentrations of pyrenocine A and pyrenocine B were *ca.* 4 and 3.2 mg per liter of culture filtrate respectively. In contrast, following culture for the same time period on CD medium, neither pyrenocine A nor pyrenocine B were detected. After incubation in SEM for a further 7 days the concentrations of total pyrenocines had declined, to give values of 1.7 and 1.6 mg per liter for compounds I and II respectively. In neutral aqueous solution the conversion of pyrenocine A to pyrenocine B is known to occur via a Michael addition of H₂O across the 8,9 double bond of 1, resulting in an equilibrium mixture of the two compounds, of which pyrenocine A predominates [14, 17]. We observed that when our isolate was grown in CD medium for a period of 4 weeks, only pyrenocine B was detected (Table II). In this medium the pH of the culture was found to decrease to *ca.* 4.5. Under these conditions the above Michael addition to form II is favoured. Subsequently, by adjustment of the culture filtrate to pH 7.0 prior to extraction (see Experimental), the addition reaction is effectively quenched. This could account for the fact that pyrenocine A (I) was not

Table II. Mycelial growth, pH of culture filtrate and yield of pyrenocine A (I) and pyrenocine B (II) on two different media.

Medium*	Growth [dry wt., gm/l]	pH	Vol. of culture filtrate [ml]	Yield [mg/l]	
				I	II
CD(A) ⁺	4.9	4.6	70	—	—
CD(B)	9.7	4.5	73	—	Tr
SEM(A)	2.6	7.8	79	4.0	3.2
SEM(B)	2.4	8.3	76	1.7	1.6

* The fungus was cultured on both modified Czapek Dox broth [CD] and sunflower extract medium [SEM] for periods of 21 days (A) and 29 days (B). For description of media see text.

⁺ = mean of 3 experiments.

— = not detected.

Tr = trace.

observed to co-occur with its water adduct (**II**) following fermentation in CD medium. An alternative explanation is that pyrenocine B is a *bona fide* fungal metabolite, as opposed to being derived solely from I by non-enzymic means, the biosynthesis of which is proportionately enhanced by this particular medium. Notwithstanding, the much greater production of both compounds in the medium containing sunflower leaf extract (SEM) as compared with the presence of only trace amounts of pyrenocines following growth on CD medium is a striking result. The effect of components from higher plants on the metabolism of microorganisms with which they interact in nature has, however, been reported previously (e.g. [18–21]).

The production of the same uncommon metabolite (pyrenocine A) by three different fungal species belonging to unrelated genera, namely *Pyrenochaeta* (Sphaeropsidales), *Penicillium* (Eurotiales) and the present *Alternaria* species (Dematiaceae) is somewhat unexpected, although analogous phenomena have been described. For example, the production of the cephalosporin antibiotics by species of *Streptomyces*, *Aspergillus* and *Cephalosporium* is known [22]. This situation is postulated not to result from parallel evolution, but rather to be due to 'promiscuous' transfer of genetic material between organisms which would be incompatible in more established gene transfer mechanisms. Methods of indiscriminate gene transfer postulated by Luckner [22] include (protoplast) fusion, and various genetic vectors such as plasmids and phages. The transfer of the genes required for pyrenocine A biosynthesis between *Alternaria*, *Pyrenochaeta* and *Penicillium*, or certain of their ancestral forms, is an intriguing possibility.

Experimental

Isolation and characterization of the fungus

Pieces of *H. tuberosus* leaf tissue at the perimeter of necrotic lesions and beyond were excised and washed thoroughly with sterile water prior to plating on V-8 juice agar. The plates were incubated at 28 °C to reveal dark-pigmented filamentous growth after 48 h. Sub-cultures from this growth on the same medium were free of bacterial contaminants and axenic. Incubation to induce sporulation was performed under continuous illumination. Conidial

morphology was examined microscopically using wet mounts at magnifications of 150X and 600X.

Fermentation and isolation of the metabolites

Sunflower extract medium (SEM) contained the hot aqueous extract of 100 g fresh weight of sunflower leaves and 5 g of sucrose per L of medium. Modified Czapek Dox broth had the composition as given in [12]. Batches of 100 ml of medium in 500 ml Erlenmeyer flasks were inoculated with an agar plug from a culture growing on V-8 juice agar. Liquid cultures were incubated at 28 °C on a gyratory shaker (170 rpm.) for 21 or 29 days. The mycelial mat was removed and weighed after drying, but was not extracted. The culture filtrate was adjusted to pH 7.0 prior to extraction (X2) with equal volumes of diethyl ether. The organic phases were combined, dried over anhydrous Na₂SO₄ and reduced to a small volume for TLC (Merck, silica gel, 0.25 mm, Et₂O). R_f **I** = 0.31, R_f **II** = 0.11. Compounds were eluted with CHCl₃. HPLC was performed using a Waters Data- and Z-Module system equipped with a reverse phase C₁₈ μ Bondapak column (5 μ); MeCN–H₂O, 6:4 at a flow rate of 1.0 ml/min, UV detector (Waters model 481) at 260 nm. Mass spectra were recorded on a VG 7070E, and NMR spectroscopy was performed on a Varian XL 300 instrument.

Bioassays

(i) Leaves

Plants were grown in a greenhouse under standard conditions at 26 °C day/18 °C night with a 16 h photoperiod. Leaves were excised and placed on moist filter paper in Petri dishes. Pyrenocine A, prepared at various concentrations in 2% aqueous DMSO, was applied to the adaxial leaf surface as 10 μ l droplets, either over needle punctures or to the intact cuticle. Control leaves were treated with 2% aqueous DMSO in the presence and absence of a needle puncture. Petri dishes were sealed to prevent desiccation of the leaf tissue and the applied droplets. Treated leaves were incubated at 24 \pm 2 °C with *ca.* 12 h photoperiod, and examined daily for development of symptoms.

(ii) Protoplasts

Sunflower protoplasts for bioassay were prepared from cotyledons of aseptically grown plants. The excised tissue was preplasmolyzed for 6 h in a hyper-

tonic solution, the main ingredient of which was sucrose at a concentration of 102.6 g/l, together with mineral salts, vitamins and hormones. Plasmolyzed tissue was digested with a mixture of cellulase and meicellase (Meiji Seika Kaisha Ltd., Tokyo, Japan) for 16 h at *ca.* 25 °C. Isolated protoplasts were suspended in a modified Murashige-Skoog medium at a concentration of 10⁵ ml. Solutions of pyrenocine A in aqueous DMSO were added to protoplast suspensions to give a final DMSO concentration of 0.15%. Control protoplast suspensions were incubated in 0.15% aqueous DMSO. After incubation at 22 °C

for 4 days protoplast viability was determined by the fluorescein diacetate staining technique as described in [15]. Viability for each pyrenocine A treatment was expressed as a percentage of the control value.

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