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Aleuria aurantia – indole metabolites of fruit bodies, mycelial culture and culture medium

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The aim of present study was to investigate and compare indole metabolites of fruit bodies, mycelium cultivated *in vitro* and culture medium of the fungus *Aleuria aurantia* (Fr.) Fuck. By use of a number of chromatographic and spectroscopic methods several indole metabolites have been detected and identified, among others the 3-indolebutyric acid was produced and extracted to the culture medium. Furthermore 3-indoleacetonitrile and tryptophane degradative products have been found both in fruit bodies and mycelium.

Key words: *Aleuria aurantia*, fruit bodies, mycelial culture, indole metabolites, 3-indolebutyric acid.

INTRODUCTION

Aleuria aurantia (Fr.) Fuck. is a fungus belonging to the family *Aleuriaceae* and the class *Ascomycetes*, which fairly commonly occurs in Poland (Gumińska and Wojewoda 1983). It forms characteristic cup-shaped fruit bodies, vividly orange in colour (Fig. 1) due to the presence of carotenoids: aleuriaxanthine and its derivatives, among others. Other chemical components of fruit bodies have not been studied so far. There have been published only several reports concerning fucose-dependent lectin, which is important for histological cell diagnosis, including early detection of neoplastic processes in hepatocytes (Fukomori et al. 1990).

Mycelial culture of this species, which can be potential alternative source of the analysed compounds (Kohlmünzer 1992) has been established for the first time in our laboratory. It was the object of preliminary phytochemical studies conducted at our Department, which were aimed to determine the -contents of metabolites, mainly fatty acids, sterols, sugars,

amines and amino acids (Węgiel and Kohlmünzer 1998). The presence of unidentified indole compounds was also demonstrated.

The objective of the present study was the comparative analysis of indole metabolites produced by fruit bodies and mycelial cultures of this species, based mainly on chromatographic and spectral methods.

MATERIAL AND METHODS

The study was conducted on fruit bodies, mycelia from *in vitro* cultures and culture medium.

- Fruit bodies of *Aleuria aurantia* were collected by M. Wawrzkiewicz in apple orchard at Mstów near Częstochowa in October 1998. They were dried at a temperature of 60±2°C and crushed before extraction;
- Mycelium was cultured *in vitro* according to the procedure described previously (5), filtered off the medium, rinsed with distilled water and freeze-dried;
- Culture medium after *in vitro* culture was concentrated (under reduced pressure) and extracted.

Mycelial culture of *Aleuria aurantia* was maintained on the modified medium, according to Oddoux (1960). To establish the culture, fragments of hymenial part of fresh fruit bodies, were inoculated after disinfection with 70° ethanol. The cultures were maintained on solid and liquid media. Culture medium was sterilised in autoclave at a temperature of 120°C, under a pressure of 1 atm. for 20 minutes.

Solid culture (Fig. 2). The sterilised medium was poured into Petri dishes 5 cm in diameter under sterile conditions. The culture was maintained in thermostat at a temperature of ±25°C for 20 days in the darkness. To obtain appropriate quantities of the material, several subcultures were conducted.

Liquid culture (Fig. 3) was maintained in Erlenmayer flasks in a fermenter. After sterilisation of the medium (under conditions described above), its 150 ml aliquots were poured to 500 ml Erlenmayer flasks and inoculated with the material derived from solid culture. The culture was shaker at room temperature under day/night for 3 weeks. Mycelium growth was characterised by 3 phases as described previously (Kohlmünzer, Węgiel and Grzybek 1998).

Extraction of indole metabolites. After defatting with petroleum ather, fruit bodies and mycelia were extracted with methanol at a temperature of 20±1°C. Dry extracts for chromatographic studies were obtained after distilling off methanol (under reduced pressure). Culture medium was extracted with ethyl acetate (1:1) at three pH variants: pH 3.0; pH 7.0 and pH 11.0. Organic phase was separated, dried with

Fig. 1. *Aleuria aurantia* – fruit bodies

Fig. 2. Static mycelial culture of *Aleuria aurantia*

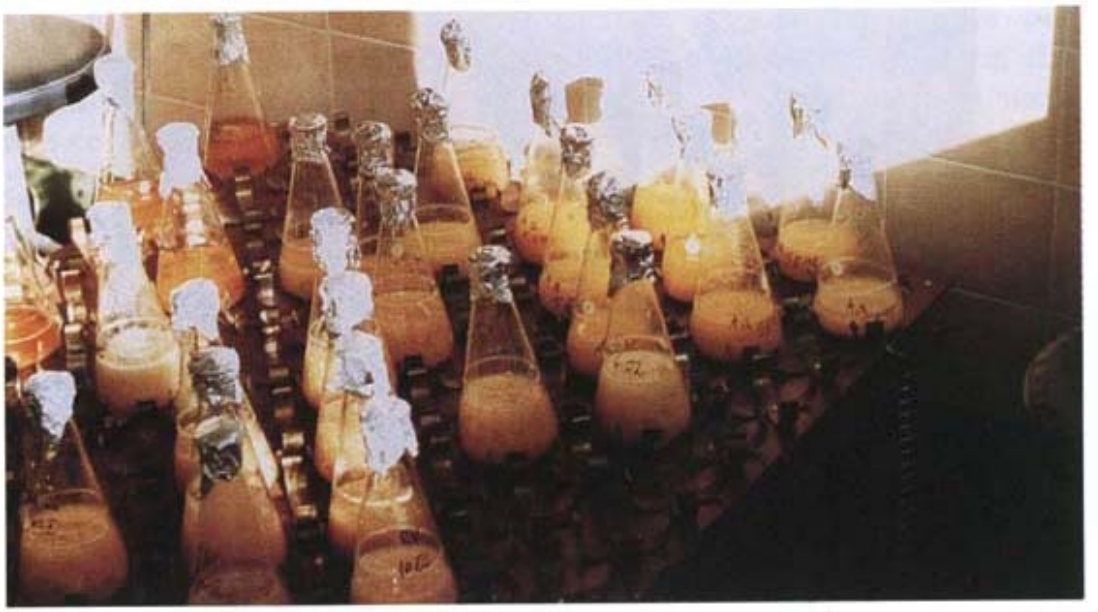

Fig. 3. Shake mycelial culture of *Aleuria aurantia*

anhydrous Na_2SO_4, concentrated (under reduced pressure) and subjected to chromatographic analysis. Fresh medium, which was not used to maintain the culture was used as the control.

Chromatography. Thin-layer chromatography (TLC). Aluminium plates covered with silica gel (Merck, DC Alufolien 60p.) measuring 20 × 20 cm were used. Developing systems:

I. n-butanol-acetic acid-water 12:3:5 (v/v/v)
II. isopropanol-ammonia-water 8:1:1 (v/v/v)

Chromogenic reagent (DAB): solution of p-dimethylbenzaldehyde in 25% HCl, freshly prepared, diluted with acetone 1:4 (v/v) before use. Spots of indole compounds were observed after spraying the plates using electronic apparatus (Sprühgerät Merck) immediately and after 24 h. Spot colours: blue, violet or yellow and yellow-orange.

Paper chromatography (PC). Whatman paper No 3, developing system I (described above). Descending chromatography; Chromogenic reagent (described above).

Preparative thin-layer chromatography (PTLC). After finishing thin-layer chromatography (TLC), the spots showing positive reaction with DAB reagent were cut off the plates and extracted with methanol. Subsequently, the solutions of indole compounds were studied using HPLC, and then with spectral methods. Authentic reference standards of indole compounds were used for preliminary identification of the isolated compounds.

Column chromatography (CC). Column was filled with silica gel (200–300 mesh) suspended in n-hexane. Compounds were eluted using solvent mixtures with increasing polarity (n-hexane-chloroform-water). The obtained fractions were analysed using TLC, 2D-TLC, PC.

High-performance liquid chromatography (HPLC). The studies were conducted using: HITACHI system equipped with a pump A: L-7100, column RP18, and solvent system: methanol 80 V%, H_2O 16.6%, CH_3COONH_4 3.4%, with detection at $\lambda = 280$ nm, in isocratic system. The 20 μl samples dissolved in methanol were injected into the column.

Spectral methods UV spectra were obtained using UV VIS Cary spectrophotometer (Varian). Absorbance of the samples dissolved in methanol (analytical grade) was measured in wavelenght range λ 220–500 nm. Specific absorbance of indole compounds was determined in wavelenght range λ max 220–280 nm. Mass spectra (EIMS) were measured using Finningan Mat 95 F system at 70 eV. Fractions or substances which showed λ max 220–280 nm in UV spectra were analysed with this method. Among other features, fragmentation ions m/z 115, 130, 135, 143, 157, 204 indicated the presence of indole compounds.

RESULTS

The application of the aforementioned, different chromatographic techniques allowed to demonstrate and preliminarily characterise indole metabolites in three study objects, namely fruit bodies, mycelium cultured *in vitro* and culture medium (Table 1).

Table 1
Indole metabolites of *Aleuria aurantia*

Fruit bodies			Mycelium	Culture medium
	Extracts			
	methanol	water		
Tryptophane	+ TLC Rf-0.43 (I) v Rf-0.35 (II) v PC Rf-.31 (I) v	+	+ see fruit bodies	–
Kynurenine I	+ TLC Rf-0.41 (I) yo Rf-0.27 (II) yo HPLC RT-1.93	+	+ see fruit bodies	–
3-OH kynurenine II	+ TLC Rf-0.44 (I) yo Rf-0.00 (II) yo HPLC RT-1.98	–	–	–
3-indolebutyric acid (IBA) III	(+)	–	(+)	+ TLC Rf-0.80 (I) bl UV max 213, 282 HPLC RT-2.32 EiMS m/e 203 (MS) 157, 131, 114
3-indoleacetonitrile IV	+ TLC Rf-0.89 (I) blg Rf-0.91 (II) blg	–	+ see fruit bodies	(+)?
–	TLC Rf-0.67 (I) bl		TLC Rf-0.68 (I) blv	–
–	TLC Rf-0.93 (I) v Rf-0.90 (II) v		TLC Rf-0.77 (I) bl UV fluoresc. (!) bl	–

Explanations: color of spots: bl – blue; blv – blue-violet; v – violet; blg – blue-gray; yo – yellow-orange.

Fruit bodies

Methanol extract: chromatography using solvent systems I and II (TLC) and I (PC) demonstrated the presence of 7 spots of compounds reacting wit DAB reagent. Four of them were blue or violet in colour, which is characteristic of indole compounds, tryptophane (or tryptamine) derivatives while 3 spots were yellow-orange (products of tryptophane degradation).

Preparative thin-layer chromatography (PTLC) and extraction of individual spots with methanol allowed obtaining homogenous solutions of the analysed metabolites.

The comparison with standard substances (TLC, PC, HPLC) confirmed identity of the extracted spots with $R_f = 0.41$ and $R_f = 0.44$ (TLC, solvent system I) with kynurenine (I) and 3-OH kynurenine (II), respectively.

Blue spot with $R = 0.81$ (PC, solvent system I) was identified as 3-indolebutyric acid (IBA) in spectral studies (UV, EIMS).

Furthermore, the presence of indoleacetonitryle ($R_f = 0.89$, TLC, solvent system I) and two unidentified indole compounds was demonstrated. The latter compounds were not identical with any freeze-dried water extract: contained amino acid tryptophane ($R_f = 0.43$, solvent system 1, violet spot) kynurenine known and 3-OH kynurenine.

Identity of these compounds with standard substances was confirmed by HPLC.

Mycelium

Mycelium was cultured *in vitro* with a yield of 2.0–2.5 g of dry mass/1 l of the medium.

Methanol extract: preliminary chromatographic analysis (TLC, PC) in solvent systems I and II indicated very complex composition of the metabolites, reacting with DAB reagent. Therefore, the extract was separated using column chromatography (CC), which allowed collecting 47 fractions. Identical fractions containing DAB + the compounds were combined, and fraction 17 was subjected to preparative thin-layer chromatography (PTLC). Such procedure allowed to demonstrate the presence of tryptophan and 3-indoleacetonitrile (IAN, violet spot, $R_f = 0.89$, solvent system I) and unidentified DAB + compound (blue spot, $R_f = 0.77$). Only trace amounts of 3-indolebutyric acid were detected in mycelium. The presence of abovementioned metabolites was confirmed with HPLC method by comparison with reference substances. Mycelium contained also several compounds in the nature of quaternary amines, e.g. choline.

Culture medium

Among the metabolites excreted into the medium and extracted with ethyl acetate at pH 3.0 and pH 7.0, only one indole compound was discovered and identified by cochromatography, UV spectrum and EIMS spectrum as 3-indolebutyric acid (IBA): PC: $R_f = 0.81$, DAB reagent – blue spot; HPLC: R_T 2.31, UV λm. 213, 282; EIMS m/z 203 (MS), 157, 131, 115.

CONCLUSIONS

1. *Aleuria aurantia* a higher fungus belonging to the family *Aleuriaceae* and the class *Ascomycetes* synthesises indole metabolites as well in fruit bodies as in mycelial culture and culture medium.
2. Chromatographic and partially spectral methods, applied to characterize the metabolites, allowed to demonstrate, besides tryptophane, principally the presence of the products of its degradation: kynurenine, 3-OH kynurenine (Kączkowski 1983) (mainly in fruit bodies) and biogenetic derivative 3-indolebutyric acid (IBA) (in fruit bodies, and mainly in culture medium). The latter was discovered in this species for the first time, similarly as 3-indoleacetonitrile (IAN) (in fruit bodies and mycelium).
3. Mycelium of the species under examination was cultured for the first time with a yield of 1.5–2.0 g of dry weight/1 l of the medium. It contained wider spectrum of the metabolites, including indole compounds detected in fruit bodies (except of 3-OH iynurenine). However, their amounts were lower than those observed in fruit bodies.
4. Culture medium obtained after 21-day *in vitro* culture contained 3-indolebutyric acid,which is a known stimulator of plant growth. The presence of this compound was confirmed br chromatographic (TLC, CC, HPLC) and spectral (UV, EIMS) methods.
5. Culture media of higher fungi can be a biotechnological source of bioactive metabolites, including indole derivatives.
6. The studied fungal species contained also at least 2 other metabolites, yielding coloured reaction typical of indoles, and possessing spectral features, characteristic of these compounds. They are subject of further studies.
7. No basic, bioactive indole compounds, including tryptamine and its derivatives were detected in the study material, which can confirm usable and edible properties of this fungus.

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Aleuria aurantia — metabolity indolowe w owocnikach, kulturze mycelialnej i pożywce

S t r e s z c z e n i e

Przedmiotem badań były metabolity indolowe wytwarzane przez owocniki i hodowlę mycelialną *Aleuria aurantia* (*Aleuriaceae, Ascomycetes*). Analizowano porównawczo zdolność wymienionego gatunku do biosyntezy tych związków. Stosując różne techniki chromatograficzne (PC, TLC, PTLC, HPLC) ujawniono obecność kilku indolowych związków, będących biogenetycznymi pochodnymi tryptofanu, a mianowicie: kwasu 3-indolomasłowego, 3 indoloacetonitrylu oraz produktów degradacji tryptofanu: kynureniny, 3-hydroksykynureniny. Szczególnie interesujące było wydzielanie do pożywki hodowlanej kwasu 3-indolomasłowego (IBA), znanego stymulatora wzrostu roślin. Obecność tego związku w pożywce została potwierdzona poza chromatografią — metodami spektralnymi (UV, EIMS).