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HYPAPHORINE ACCUMULATION IN HYPHAE OF THE ECTOMYCORRHIZAL FUNGUS, *PISOLITHUS TINCTORIUS*

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Key Word Index—*Pisolithus tinctorius*; Basidiomycete; ectomycorrhizal fungi; hypaphorine; tryptophan betaine; indole alkaloid.

Abstract—Tryptophan betaine or hypaphorine, is the major indolic compound detectable in free-living hyphae of the ectomycorrhizal fungus, *Pisolithus tinctorius*. Hypaphorine could not be detected in five other ectomycorrhizal species.

INTRODUCTION

Indole acetic acid (IAA) accumulation by ectomycorrhizal fungi is well documented [1]. It has been suggested repeatedly that IAA regulates ectomycorrhizae ontogenesis during root colonisation by symbiotic fungi. The concentration of IAA in fungal tissues, in pure culture on mineral nutrient media, is rather low (5.62 nmol g^{-1} fr. wt for *Pisolithus tinctorius* 441) and unknown in symbiotic tissues, the fungal sheath or the Hartig net. While studying the regulation of aromatic-compound metabolism in *P. tinctorius*/*Eucalyptus* ectomycorrhizae (to be published elsewhere), we isolated from fungal tissues a major indolic compound, the concentration of which was at least 900 times higher than that of IAA. No such molecule has been indentified in any other fungi, including ectomycorrhizal fungi.

RESULTS AND DISCUSSION

Pisolithus tinctorius mycelial mats were grown on agar medium for three weeks before analysis. A major aromatic compound was detected and purified by HPLC. Its UV spectrum (strong maximum at 279 nm with shoulders at 287 and 273 nm), as well as a violet colour with dimethyl-aminocinnamaldehyde indicated its indolic structure. It was also Dragendorff-positive suggesting it could be a quaternary nitrogen compound [2]. The ^{1}H NMR (400 MHz, D_2O) and ^{13}C NMR (75 MHz, D_2O) confirmed the indolic nature. The ^{1}H spectrum exhibits signals at 7.1–7.7 ppm (5H, *m*, aromatic H) and 3.20 ppm (9H, singlet) and the $^{13}C\{^1H\}$ spectrum shows a singlet at 55.1 ppm characteristic of the methyl signals of a $-N^+(CH_3)_3$ group. Finally, this compound was identified as hypaphorine, the betaine of tryptophan, by comparison of physical and spectral data (HPLC, UV absorption, ^{1}H and ^{13}C NMR) with those of hypaphorine purified from *Glycyrrhiza yunnanensis* roots by Ohtani *et al.* [3] and hypaphorine synthesized *in vitro* from tryptophan according to Romburgh and Barger [4].

Hypaphorine was first isolated from seeds of *Erythrina hypaphorus* [4] and has been detected in several other higher plants (*Sida cordifolia* [5], *Glycyrrhiza yunnanensis* [3], *Desmodium girans* [6], *Desmodium trifolium* [7], *Pterocarpus officinalis* [8], *Abrus precatorius* [9], *Lens culinaris* [10] and some South American Moraceae [11]) but never before from fungi or any other microorganism. Hypaphorine concentration in *P. tinctorius* mycelial mats varied 18-fold depending on strain, from 0.29 to 5.14 nmol mg^{-1} fr. wt (Table 1). The same range of variation between strains was recorded when hypaphorine concentration was related to ergosterol concentration, a sterol constituent of the fungal plasma membrane commonly used to assess fungal living biomass inside roots or in soil. The hypaphorine concentration in fungal mats range from 0.51 to 4.86 nmol μmol^{-1} ergosterol (Table 1).

High hypaphorine concentrations seem to be a characteristic of *P. tinctorius*. Indeed, hypaphorine could not be detected in five other fungal strains belonging to four ectomycorrhizal species, including strain 331, a tryptophan- and IAA-overproducing mutant of *Hebeloma cylindrosporum* [12] (detection limit in our experimental conditions, 0.05 nmol mg^{-1} fr. wt).

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Table 1. Occurrence of tryptophane betaine (hypaphorine) in ectomycorrhizal fungi hyphae in pure culture*

Fungal species	Strain no.	Hypaphorine	
		nmol mg^{-1} fr. wt	nmol μg^{-1} ergosterol
Pisolithus tinctorius	ptA	6.02 ± 1.00	2.81 ± 0.41
	441	5.14 ± 0.57	4.86 ± 0.68
	2144	4.98 ± 0.31	6.21 ± 0.88
	ptC	4.31 ± 0.29	3.40 ± 0.54
	56	3.75 ± 0.40	4.14 ± 0.54
	OSSA	3.02 ± 0.31	4.28 ± 1.93
	556	2.58 ± 0.39	4.74 ± 0.52
	1101	1.73 ± 0.22	1.65 ± 0.42
	570	1.28 ± 0.32	4.83 ± 0.86
	4461	0.60 ± 0.18	0.47 ± 0.04
	98	0.52 ± 0.11	1.75 ± 0.33
	506	0.43 ± 0.10	4.40 ± 1.33
	4320	0.29 ± 0.13	0.51 ± 0.25
Hebeloma cylindrosporum	H1	ND	ND
	331	ND	ND
Paxillus involutus	COU	ND	ND
Cenococcum geophilum	SIV	ND	ND
Scleroderma dictyosporum	ORS 7731	ND	ND
Laccaria bicolor	S238N	ND	ND

*Mean of three replicate cultures ± SD.
†ND, not determined; detection limit 0.05 nmol mg^{-1} FW.

A role for this indolic compound either during fungal growth and development or during interaction with root tips being converted to ectomycorrhizae has still to be elucidated.

EXPERIMENTAL

General. HPLC was conducted according to Ref. [13] with UV detection at 280 nm. The column was a Lichrospher RP18 endcapped (5 μm, 4 × 250 mm, Merck) using a gradient of 0–54% MeOH in water at pH 3 over 36 min with a flow rate of 1.5 ml min^{-1} (hypaphorine R_t: 14 min). Ergosterol content was estimated according to Ref. [14]. ^{1}H and ^{13}C{^{1}H} NMR expts were performed at 400 MHz and 75.5 MHz, respectively. Chemical shifts are referred with respect to the TMS signal.

Fungi. Cultures of *Pisolithus tinctorius* (Pers.) Coker & Couch (13 strains isolated in Europe, Australia, Africa or North America: 441, 2144, 1101, 4461, 4320, 570, 98, 506, 556, 56, OSSA, ptA, ptC), *Laccaria bicolor* (Maire) Orton (one strain: S238N), *Paxillus involutus* (Batsch. ex Fr.) Fr. (one strain: COU), *Cenococcum geophilum* Fr. (one strain: SIV), Scleroderma dictyosporun Pat. (one strain: ORS 7731), *Hebeloma cylindrosporum* Romagnesi (two strains: H1, 331), are maintained in the ectomycorrhizal fungi collection in the Laboratoire de Microbiologie Forestière (INRA, Nancy, France). Fungal strains were grown for 3 weeks at 25° on cellophane laid over low-sugar modified Pachlewski's medium (7.3 mM KH_2PO_4, 2.7 mM ammonium tartrate, 7.3 mM $MgSO_4 \cdot 7H_2O$, 100 mM glucose, 2.9 mM thiamine–HCl and 1 ml of a trace element stock soln (Kanieltra Co.) in 2.0% agar). For comparisons between strains, hyphae were sampled at the margin (5 mm) of the fungal colonies, hypaphorine extracted as described below and quantified by HPLC.

Isolation and characterization of hypaphorine. Mycelia of *Pisolithus tinctorius* 441 (9 g) was powdered in liquid N_2 using a mortar and pestle. Extraction solvent (MeOH–H_2O, 4:1) was added and the powdered mycelia kept at 4° for 1 hr before centrifugation at 4° (10 000 *g* for 20 min). The pellets were extracted 2 more times. The supernatant frs were comb. (30 ml) and filtered (Sartorius filter, 0.2 μm). Hypaphorine was purified by reverse-phase HPLC as previously described and collected in 4.5 ml frs. These frs were comb. (135 ml) and evapd to dryness under red. pres. at 35°. This procedure yielded *ca* 4.5 mg of product 1 suitable for analysis. UV λ_{max}^{MeOH} nm: 279, 287, 273. ^{1}H NMR (400 MHz, D_2O) δ3.20 (9H, *s*, $-N^+(CH_3)_3$), 3.28 (2H, *d*, $-CH_2CH-$), 3.87 (1H, *dd*, $-CH_2CH-$), 7.1–7.7 (5H, *m*, aromatic H). ^{13}C{^{1}H} NMR (75 MHz, D_2O) *d* 174.7 (CO_2^-), 139.4 (C-9), 129.7 (C-8), 127.9 (C-7), 125.3 (C-6), 122.7 (C-5), 121.4 (C-4), 115.3 (C-2), 110.3 (C-3), 82.1 (α-C), 55.2 ($-N^+(CH_3)_3$), 25.9 (β-C). UV, ^{1}H and ^{13}C{^{1}H} NMR spectra of **1** were identical to those of hypaphorine purified from *Glycyrrhiza yunnanensis* roots [3] and ^{1}H NMR spectra were identical to those of hypaphorine synthesized from tryptophane [4].

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