

CORTINARIUS SECTION ORELLANI: ISOLATION AND CULTURE OF *CORTINARIUS ORELLANUS*

By S. RAPIOR AND C. ANDARY

*Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie, Avenue Charles Flahault,
34060 Montpellier Cedex, France*

AND D. MOUSAIN

*Laboratoire de Recherches sur les Symbiotes des Racines, Institut National de la Recherche Agronomique,
9 place Viala, 34060 Montpellier Cedex, France*

An attempt was made to isolate the mycelium of *Cortinarius orellanus* and to look for orellanine in it, the structure of this molecule being 3,3',4,4'-tetrahydroxy-2,2'-bipyridyl-1,1'-dioxide. A mycelial isolate of *C. orellanus* was obtained for the first time from an agar medium similar in composition to Pachlewski and Oddoux media. The identity of the cultures was confirmed by the chemical characterization of orellanine by tlc on cellulose and photodecomposition under uv light. The orellanine content from the cultured mycelium was weaker than those from the carpophore. This result was compared with those obtained by other authors for the genus *Amanita* and *Gyromitra*.

After several deadly poisonings in Poland in 1952, Grzymala (1957) described the first cases of orellanine poisonings, which were attributed to *Cortinarius orellanus* Fr. (Skirgiello, 1957). Following the isolation of orellanine by Grzymala (1962), Antkowiak & Gessner (1979) continued the work and reported a bipyridine N-oxide structure for the molecule (Fig. 1), but in fact there was no basis for choosing the latter from among the other possible isomers (Rapior, 1983). At the same time, Dehmlow & Schulz (1985) and Tiecco *et al.* (1986) synthesized orellanine and confirmed the structure as that indicated by the Polish authors; it is 3,3',4,4'-tetrahydroxy-2,2'-bipyridyl-1,1'-dioxide.

Few studies have been devoted to the culturing of *Cortinarius* because of the difficulties in isolating the vegetative mycelia of these species, which are mostly mycorrhizal (Kühner, 1946, 1947). In particular, the many attempts to culture *C. orellanus* have failed (Oddoux, 1955).

In a continuation of our studies on *Cortinarius* sect. *Orellani* (Andary *et al.*, 1986), it seemed of interest to attempt to isolate the mycelium of *C. orellanus* and to look for orellanine in it, with a view to possible bioproduction of the substance.

MATERIALS AND METHODS

Obtaining mycelial thalli

Fungal material. In all the tests, *Cortinarius orellanus* Fr. was collected on siliceous soil

beneath *Quercus petraea* (Mattuschka) Liebl. (= *Q. sessiliflora* Salisb.) and *Castanea sativa* Miller (= *C. vulgaris* Lam.) in Arnac forest near Bédarieux, Hérault, France. Healthy young carpophores were harvested and thoroughly cleaned several hours before samples were taken.

Sampling. The cap was separated from the stipe and cleaned with absorbent cotton wool soaked in 95% ethanol. Fragments of the inner part of the cap tissue were removed, or lamellae accompanied by underlying zones of the subhymenium. In the latter case, the samples were dipped in 95% ethanol for 2-3 s. Explants of different sizes (3-10 mm diam) were plated on the surface of a nutrient agar medium.

Culture and isolation conditions. The samples were cultured on the following nutrient agar medium: 83.3 mM glucose, 7.4 mM-KH₂PO₄, 2 mM-MgSO₄, 2.7 mM ammonium tartrate, 3.3 mM L(-)asparagine, 0.15 μM thiamine HCl, 15 g l⁻¹ agar, 5 g l⁻¹ malt extract, 1 g l⁻¹ casein hydrolysate, 0.0005 g l⁻¹ ferric citrate, and 0.25 ml l⁻¹ trace elements (Morizet & Mingeau, 1976). The pH of the medium was adjusted to 6.3 with 0.1 M-NaOH before autoclaving for 30 min at 120 °C. This medium, designated as PO, was then distributed in sterile Petri dishes and plated. The dishes were sealed with adhesive tape and placed in the dark in an incubator at 24 °. After isolation of the mycelium, cultures were maintained by removing mycelial implants at the edge of the mother cultures and

transferring them to the PO medium described above. The isolate obtained was designated as L6 in our fungal culture collection.

Characterization of orellanine in mycelium

Extraction and purification. Extraction was done with four fresh thalli (ground to a fine powder in a mortar) macerated several times for periods of 15 min in 200 ml of 20% methanol (v/v) at room temperature in the dark under magnetic agitation. The extracts were combined and centrifuged at 3000 g (r_{av} 6 cm) for 20 min. The pellet was removed and the supernatant evaporated nearly to dryness in a rotary vacuum evaporator, after which it was resuspended in boiling 20% methanol (v/v). The volume was then adjusted to 5 ml with the same solvent.

The resulting extract was purified by passage through a 22 × 200 mm column containing Amberlite XAD-2 resin (ref. 06443, Fluka). Elution was carried out by successive passages of the following solvents: water, methanol/water (1:1, v/v), methanol. Twenty ml fractions were then collected.

Characterization. Orellanine was evidenced by tlc on cellulose (ref. 5716, Merck) developed in three different solvents: N-butanol/hydrochloric acid/chloroform/water (40:20:15:3.8, by vol. = BCCE); N-butanol/acetic acid/water (40:10:22, by vol. = BAW); isopropanol/hydrochloric acid/water (85:22:18, by vol. = ICW) and by a specific photodecomposition under uv light (360 nm) according to a previously described method (Andary *et al.*, 1986).

RESULTS

The isolated mycelium grew to 5 cm in diam in 28 d. The culture was uniformly circular, with clear contours. The mycelium was aerial, with a cottony appearance, and was very dense, but did

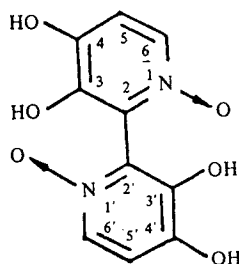


Fig. 1. Orellanine, or 3,3',4,4'-tetrahydroxy-2,2'-bipyridyl-1,1'-dioxide.

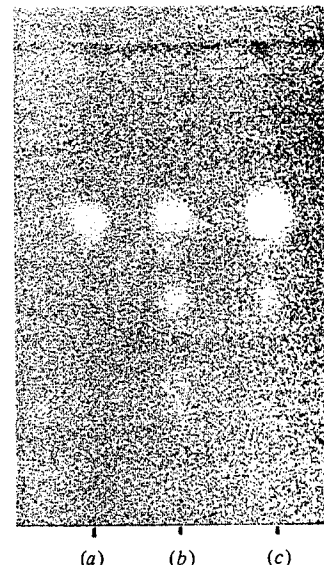


Fig. 2. Tlc on cellulose of: (a) an authentic sample of orellanine; (b) an extract of *C. orellanus*; (c) a purified extract of thalli of *C. orellanus* isolate L6 cultured in vitro.

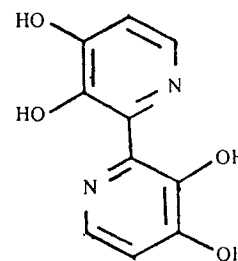


Fig. 3. Orelline, or 3,3',4,4'-tetrahydroxy-2,2'-bipyridyl.

not penetrate the agar. Its colour was initially white and did not change with age; by contrast, the reverse had an ochraceous tinge.

Microscopic observation revealed many very thin hyphae (1.5 μm diam), which were flexuous, and either hyaline or granular and refringent. They were not very ramified, and apparently had neither septa nor clamp connexions. We also occasionally observed thicker hyphae (2.5 μm diam), which were shorter, granular and had a very small number of clamp connexions.

Purification of a raw extract of mycelium involves eliminating pigments in the aqueous and hydromethanolic fractions, and isolating orellanine in the last methanolic fractions. Tlc on cellulose and development in the three above-mentioned

solvents (in BCCE, $R_f = 0.5$; in BAW, $R_f = 0.75$; in ICW, $R_f = 0.79$) did in fact reveal that the methanolic fraction contained orellanine (Fig. 2). The compound appeared characteristically in the form of a dark spot which, after 1–3 min exposure to uv light, produced a bluish-white fluorescence due to its photodecomposition. This photodecomposition was previously shown (Andary *et al.*, 1986) to be a facile and specific orellanine decomposition, by the uv light, into the N-deoxidized molecule, i.e. orelline or 3,3',4,4'-tetrahydroxy-2,2'-bipyridyl (Fig. 3).

DISCUSSION

Our preparation of pure cultures of *C. orellanus* was inspired by the method of Kühner (1946, 1947), which was taken up again and complemented by Oddoux (1955), whose experiments concerned more than 600 species of fungi, including about 70 *Cortinarius* spp. Taking into account the strict nutritive requirements of the Homobasidiomycetes and several positive results obtained by Oddoux (1955) in isolating certain *Cortinarius* spp., we developed the PO medium which is capable of satisfying the needs of *C. orellanus*. The medium is based on the composition of Pachlewski (1967) and Oddoux (1955) media. The carbohydrate sources represented by glucose and malt (rich in maltose) are very favourable for mycelial growth (Oddoux, 1955). Nitrogen is provided in mineral form (neutral ammonium tartrate) and organic form (L(-)asparagine, casein hydrolysate).

On the basis of many tests, it appears that the developmental capacity of *C. orellanus* is not only related to the culture medium, but also directly depends on the conditions of health and maturity of the specimen chosen. This is why mycelial hyphae only appeared with young and very fresh carpophores. Moreover, the cultures were only prepared from fragments of lamellae comprising the hymenium along with a portion, variable in size, of the underlying regions of the subhymenium. With this method, the origin of the mycelium cannot be known with certainty; it could be the subhymenial structures or the spores themselves. The growth of the mycelium depends not only on the age and cleanness of the hymenophore, which is checked under binocular magnifying glasses, but also on the time elapsed between collecting and plating, which must not exceed 2 h. Furthermore, only implants of medium size (6 mm diam) can produce cultures with a minimum risk of contamination.

The originality of this work is primarily related to the culturing conditions, which allowed us to obtain, for the first time, a mycelial isolate of *C.*

orellanus. Moreover, although microscopic observation only revealed the classic elements of a vegetative mycelium, the identity of the cultures was confirmed by the chemical characterization of orellanine. This molecule was specifically evidenced by photodecomposition, a simple technique carried out on tlc chromatograms (Andary *et al.*, 1986). Since the possibilities for determining a species by its vegetative mycelium are very limited, this chemical method is valuable.

It should be noted that the orellanine content of the mycelium obtained under the above conditions is very low compared to that of the carpophore. The extracts used for analysis were enriched by passage over adsorbent resin. Secondary metabolites detected in carpophores are usually found at different concentration in cultured mycelia, depending on the species and the culture conditions (media, age of the culture). For instance, amanitines (toxic cyclopeptides) were not found in the cultured mycelia of American toxic *Amanita* spp. (*A. verna* (Bull.) Murr., *A. tenuifolia* (Murr.) Murr., Stark & Kimbrough, 1973) or *Galerina marginata* (Fr.) Kühn. (Andary & Oddoux, unpubl.). In another case, very small quantities of bufotenin were found in the mycelia of *Amanita citrina* (Schaeffer) Roques cultured in vitro (0.03% of dry matter, Tyler & Groger, 1964). The carpophores of the latter species are, by contrast, more rich in bufotenin (0.6% of dry matter, Andary *et al.*, 1978). In *Gyromitra esculenta* (Pers.) Fr., however, the methylhydrazine concentration (in the form of hydrazones variably chelated in the fungus) was almost identical in the cultured mycelium and in the carpophore (Raudaskoski & Pyysalo, 1978).

By obtaining *C. orellanus* cultures on a solid medium, we have been able to begin physiological studies of the mineral nutrition (nitrogen and phosphate nutrition) of thalli cultured on unagitated liquid nutrient media (Rapior *et al.*, unpubl.).

These data are a useful basis for work on the nutritional needs of thalli and for studies on the precursors of orellanine synthesis in mycelia. Such metabolic studies should make it possible to develop large-scale bioproduction of orellanine.

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