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INFLUENCE OF NITROGEN SOURCE ON GROWTH OF *CORTINARIUS ORELLANUS* AND ON ACCUMULATION OF NITROGEN AND PHOSPHORUS IN MYCELIUM

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The influence of nitrogen source (nitrate, ammonium, nitrate and ammonium, glutamine) on growth of *Cortinarius orellanus* (isolate L6) and on accumulation of nitrogen and phosphorus in the mycelium was studied in vitro. Growth was poorest on nitrate which showed no significant accumulation, as in other Homobasidiomycetes previously studied. Soluble organic nitrogen and amine nitrogen accumulated in greater quantities (μmol N per mycelium) and concentrations (μmol N g^{-1} D.W.) on nitrate medium. The relative proportions of insoluble nitrogen with respect to total nitrogen reached 75 and 40 % on ammoniacal and nitric media, respectively. This results from those obtained in other Homobasidiomycetes and in most of the cultivated Phanerogams. This could reflect differences in ways of assimilating ammonium in vascular plants and indicates the role of ammonium in the enzymatic mechanisms that regulate amino-acid synthesis. The mean concentrations of total phosphorus varied from 0·8 to 0·9 % of the dry weight of the mycelium.

In a previous study (Rapior *et al.*, 1987), we isolated and cultured a strain of *Cortinarius orellanus* designated as L6. The identity of the culture was confirmed by the chemical characterization of orellanine, a toxic molecule assumed to be responsible for orellanine poisoning (Andary *et al.*, 1986). These results (Rapior *et al.*, 1987) were indispensable for research on the nutritional needs of the mycelium and for studies on the precursors of orellanine synthesis.

The nitrogen (inorganic and organic) source in the culture medium is one of the important factors in mycelial production. We therefore studied the separate and concomitant nutritive influence of inorganic nitrogen ions (NO_3^-, NH_4^+) and that of an amide (glutamine) on the mycelial mass yielded in vitro. We particularly wished to determine whether *C. orellanus* used NO_3^-, since the culture medium for this species contained only reduced nitrogen (Rapior *et al.*, 1987). Moreover, there are few published data on the inorganic nutrition of Homobasidiomycete mycelia (Salsac *et al.*, 1982; Mention & Plassard, 1983; Mousain & Salsac, 1984). For this reason, we assayed different forms of nitrogen and also total phosphorus in the *C. orellanus* mycelia.

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MATERIALS AND METHODS

Cultural conditions

The L6 isolate of *Cortinarius orellanus* Fr. (Rapior *et al.*, 1987) was cultured on a medium containing: glucose, 55·6 mM; KNO_3, 6 mM; NaH_2PO_4, 2 mM; $CaCl_2$, 1 mM; $MgSO_4$, 1 mM; thiamine HCl, 0·3 μM; ferric citrate, 0·0005 %; microelements, 0·2 ml l^{-1} (Morizet & Mingeau, 1976); agar, 1·5 %. Mycelial plugs (8 mm diam) removed from the edge of colonies were transferred onto 100 ml unagitated liquid medium (culture method according to Mention & Plassard, 1983). The basic medium contained: glucose, 55·6 mM; $CaCl_2$, 1 mM; $MgSO_4$, 1 mM; thiamine HCl, 0·3 μM; ferric citrate, 0·0005 % and microelements, 0·2 ml^{-1} (Morizet & Mingeau, 1976). The nitric medium (Ni) also included: KNO_3, 6 mM and NaH_2PO_4, 2 mM; the ammoniacal medium (A): NH_4Cl, 6 mM; KH_2PO_4, 2 mM; KCl, 2 mM; K_2SO_4, 1 mM and NaCl, 0·2 mM; the mixed medium (NiA): KNO_3, 4 mM; NH_4Cl, 2 mM; NaH_2PO_4, 2 mM and KCl, 2 mM; and the organic medium (Glm): NaH_2PO_4, 2 mM; KCl, 6 mM and filter sterilized glutamine, 3 mM (pore diam 0·2 μm). The pH of the media was adjusting to 6·0 with 0·1 M-NaOH before autoclaving for 30 min at 115–120 °C. Cultures were incubated at 24° ± 1° for 28 d. Eight

Table 1. *Growth of* C. orellanus *mycelium (mg) and mean concentration of nitrogen in various forms (μmol N g^{-1}* D.W.*) in the mycelium of* C. orellanus *cultured on different nitrogen sources. (Data expressed as mean ± 95 % confidence interval)*

	Growth and nitrogen forms	Medium			
		Ni	A	NiA	Glm
Inorganic-N	D.W.	114±63	282±21	206±29	235±38
	Ammonium-N	1·5±1·5	1·4±1·2	1·3±0·5	2·2±3·4
	Nitrate+Nitrite-N	12±0·9	—	1·0±0·4	—
Soluble organic-N	Amide - N	27±6	7±12	28±12	31±4
	Amine - N*	1486±413	385±79	836±144	835±267
	Total sol. - N	1515±406	393±66	866±132	868±260
Insoluble organic - N	Chitin - N	162±57	158±29	116±20	101±17
	'Proteic' - N†	846±661	996±732	1210±512	570±700
	Total insol. - N‡	1008±604	1154±703	1326±492	671±683
	Total - N	2523±198	1547±637	2192±360	1539±423

* Amine - N = (Soluble organic-N) - (Amide-N+Ammonium-N).
† 'Proteic'-N = (Total insoluble-N) - (Chitin-N).
‡ Total insoluble - N = (Total-N) - (Soluble organic-N).

replicates of each culture were used. After 28 d, the pH of the medium was: 4·1±0·1 (Ni); 2·6±0·1 (A); 2·8±0·02 (NiA); 3·1±0·2 (Glm).

Assays

The D.W. of the mycelia was obtained by washing with an isotonic solution of glucose followed by filtration through 'Viledamop' sheet and lyophilization followed by weighing.

Soluble forms of nitrogen (nitrate, amino acids, amides) were assayed in extracts obtained by treatment of total culture biomass with 10 ml 0·1 M-HCl. Nitrate was reduced to nitrate by passage through a cadmium column; nitrite formed by reduction and pre-existing nitrite were assayed colorimetrically after diazotization (sulfanilamide and N-1 naphthyl ethylene diamine dichloride). After Kjeldahl mineralization of the acid extract and mycelial dry matter, the soluble organic nitrogen and total nitrogen were determined by ammonium assay with Nessler's reagent; NH_4^+ and amides were assayed after release of ammonia in an alkaline medium in Conway cells (Conway, 1958). Chitin nitrogen was estimated after acid hydrolysis of mycelia and colorimetric assay of glucosamine residues (Tsuji *et al.*, 1969; Vignon *et al.*, 1986). Total phosphorus was determined after mineralization with 60 % perchloric acid and colorimetric assay according to Taussky & Shorr (1953).

The results were subjected to analysis of variance and a Duncan Multiple Range test (Beyer, 1968). The concentrations of amide, amine, total insoluble and 'proteic' nitrogen are calculated as the differences of the means of other assay results (Table 1). Student *t*-tests were carried out to obtain 95 % confidence intervals.

RESULTS

Mycelial growth

The dry weights produced by 28 d growth of cultures are shown in Table 1. On Ni medium, the mean D.W. was 114 mg. This was significantly lower than those obtained on media containing reduced nitrogen, which ranged from 206 to 282 mg. Growth was best on A medium.

Although nitrate was found to be a less favourable nitrogen source than reduced nitrogen, growth on the Ni medium was significant despite high variability on this medium. This confirms the results of previous studies on some 20 species of Homobasidiomycetes (Salsac *et al.*, 1982; Mention & Plassard, 1983) and indicates that isolate I.6 is able to assimilate NO_3^-.

Accumulation of nitrogen in mycelia

The concentration of total nitrogen in mycelia cultured in the presence of nitrate (Ni, NiA) was significantly higher than in mycelium cultured on reduced nitrogen (A, Glm) (Table 1). This was due to reduced growth of mycelium on these media, since the quantity of nitrogen accumulated per mycelium did not differ significantly between the media (Table 2). This result is not in agreement with those from other Homobasidiomycetes, which indicate a consistently higher total nitrogen content in mycelia cultured on ammonium, com-

Table 2. *Quantities of total and organic nitrogen accumulated in the mycelium of* C. orellanus (*μmol N per mycelium*). (*Data expressed as mean* ± 95 % *confidence interval*)

Medium	Total nitrogen	Soluble organic nitrogen
Ni	442 ± 35	265 ± 71
A	435 ± 26	111 ± 14
NiA	443 ± 107	181 ± 51
Glm	391 ± 50	217 ± 34

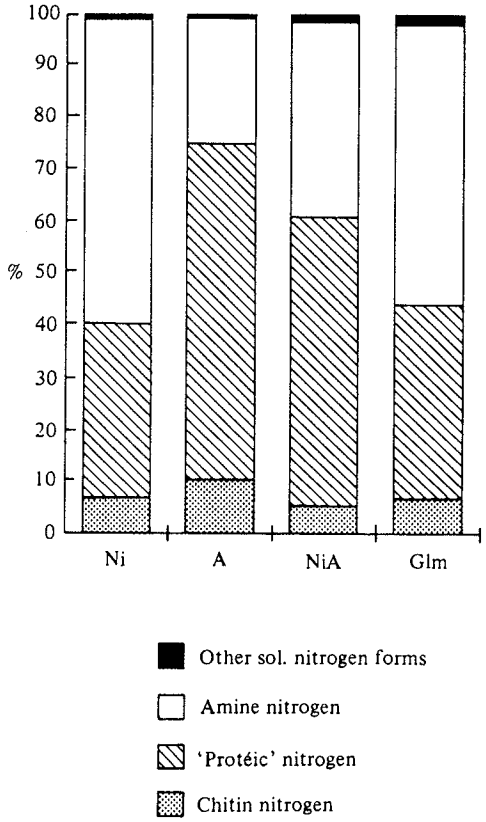

Fig. 1. Percentages of nitrogen in different forms with respect to total nitrogen in the mycelia of *C. orellanus*.

pared to those cultured on nitrate (Salsac *et al.*, 1982; Mention & Plassard, 1983). Nevertheless, the levels of total nitrogen in *C. orellanus* (2·2–3·5 % of the D.W.) are of the same order of magnitude as those measured in other Basidiomycetes (Mention & Plassard, 1983) and in most of the higher plants (see for example Mengel & Kirkby, 1979).

The soluble organic nitrogen concentration was highest in mycelia cultured on Ni medium. The A medium resulted in the lowest mean value (Table 1). The differences between the Ni and A media were not due to reduced growth of the mycelia on Ni: the quantity of soluble organic nitrogen accumulated per mycelium was also larger on Ni than on A (Table 2). The differences between the Ni and A media are futher illustrated in Fig. 1, showing the relative proportions of nitrogen in different forms compared to total nitrogen (60 % soluble nitrogen on Ni, 25 % on A). This result differs from the data of Mention & Plassard (1983), which indicate that the mycelia of *Suillus luteus* and *Pisolithus tinctorius* accumulate more soluble organic nitrogen on an ammoniacal medium than in the presence of nitrate.

More than 96 % of the soluble organic nitrogen was in the form of amines (Table 1), and relative concentrations of soluble organic nitrogen and amine nitrogen were similar on the different media. The following three groups differed with respect to their amine nitrogen concentrations, in descending order: Ni; NiA and Glm; A. As in other Homobasidiomycetes studied (Mention & Plassard, 1983), there was no accumulation of NO_3^-; practically all of the nitrate absorbed was assimilated. Absorption of NO_3^- by the mycelia is not very high; the reported mean net fluxes are no greater than 1 to 2 μmol h^{-1} g^{-1} fresh weight, which is 5–10 times lower than those measured in the roots of cultivated Phanerogams (see for example Vignon, 1984). Consequently, the ammonium arising from the reduction of this nitrate, which never accumulates (see Table 1), is always at a low concentration in the cytoplasm. This probably leads to a weak glutamate dehydrogenase (GDH) activity, since the K_M of this enzyme is relatively high, i.e. of the order of several mM (Miflin & Lea, 1980). According to Martin (1986), GDH is essentially responsible for the assimilation of ammonia in the Basidiomycetes. If the activity of this enzyme is limited by the low NH_4^+ concentration (arising from the reduction of NO_3^-), the formation of glutamic acid (Glu), which is the substrate of many transaminases, could become a factor limiting proteosynthesis. On the other hand, the activity of glutamine synthetase (GS), which has a high affinity for NH_3 of the order of 10 μmol l^{-1} (Saint John *et al.*, 1985), would remain strong, and this could result in an accumulation of a fimited number of nitrogenous molecules derived from glutamine (Gln). By contrast, on A medium, the influx of ammonium, by passive diffusion could be large enough to allow suitable GDH activity and consequently a sufficient synthesis of glutamate and proteins (Fig. 2).

The mean concentration of total insoluble nitrogen was highest on NiA and lowest on Glm

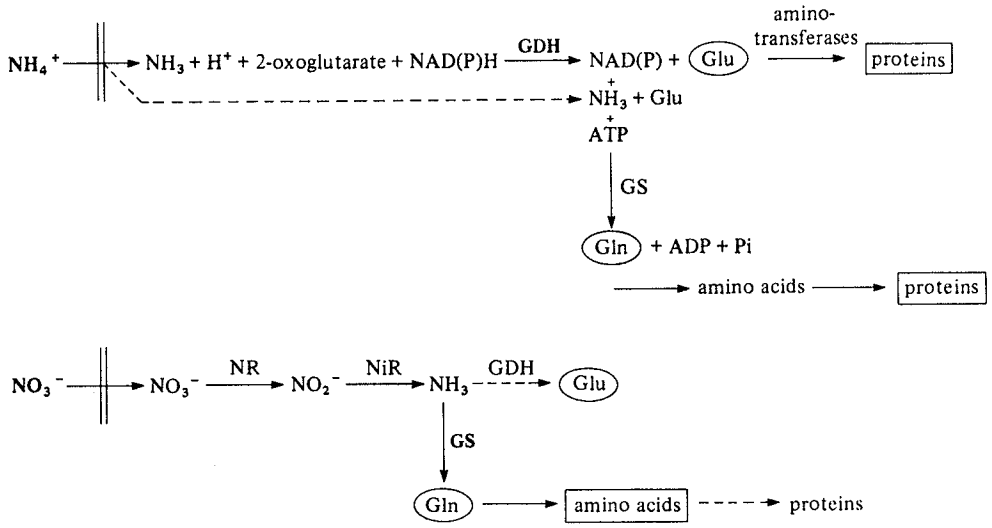

Fig. 2. Proposed pathways of ammonium and nitrate assimilation by *Cortinarius orellanus*. NR, nitrate reductase; NiR, nitrite reductase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; Glu, glutamic acid; Gln, glutamine.

Table 3. *Total phosphorus accumulation in* C. orellanus *mycelium. Total phosphorus concentrations are expressed as* μ*mol* $P_i\,g^{-1}$ D.W. *Total phosphorus quantities are expressed as* μ*mol* P_i *per mycelium. (Data expressed as mean* $\pm$ *95 % confidence interval)*

Medium	P concentration	Quantity of P per mycelium
Ni	376 ± 77	52 ± 30
A	261 ± 14	73 ± 6
NiA	393 ± 63	82 ± 23
Glm	310 ± 46	71 ± 16

(Table 1). The proportion of insoluble nitrogen with respect to total nitrogen reached 75 % on the A medium, but only amounted to 40 % on the Ni medium (Fig. 1), which is in agreement with the hypothesis stated above.

Chitin nitrogen amounted to less than 9 % of the total insoluble nitrogen on NiA and between 13 and 16 % on the other media (Fig. 1). The percentages of chitin nitrogen with respect to total nitrogen were lower (using the same method) than those measured in *Suillus luteus*. *Pisolithus tinctorius*, and *Hebeloma cylindrosporum* cultured on Ni, and also lower than those measured in *P. tinctorius* and *H. cylindrosporum* cultured on A (Mention & Plassard, 1983). Irrespective of the culture medium, the quantity of NH_4^+ accumulated and its proportion relative to total nitrogen (0·1 %) were very low (Table 1).

Assays of $NO_3^- + NO_2^-$ in mycelia cultured on Ni and NiA showed negligible concentrations, i.e. less than 0·05 % of total nitrogen, which confirms the findings that nitrate accumulation is negligible in the Homobasidiomycetes, unlike most of the cultivated Angiosperms (Mention & Plassard, 1983).

Accumulation of phosphorus in mycelia

The mean concentration of total phosphorus ranged from 261 to 393 μmol $P_i\,g^{-1}$ D.W., i.e. 0·81–0·91 % of the D.W. (Table 3). These values are close to those measured in the mycelia of *Pisolithus tinctorius* cultured for 21 d at 32° on Ni, A, and NiA, which ranged from 302 (NiA) to 356 (Ni) μmol $P_i\,g^{-1}$ D.W. (Mousain, unpubl.). In the mycelia of *C. orellanus*, the total phosphorus concentration was significantly higher on the two media containing nitrate than on those containing only reduced nitrogen. This can be attributed to the reduced growth of mycelia on the Ni and NiA media. The quantities of phosphorus accumulated per mycelium did not differ significantly with different nitrogen sources (Table 3).

DISCUSSION

Comparison of the growth of *C. orellanus* on different nitrogen sources indicates that this species is able to assimilate nitrate, like many other species of Homobasidiomycetes previously studied (Mention & Plassard, 1983; Plassard *et al.*, 1986). Nevertheless, reduced nitrogen sources (ammonium or glutamine) were more favourable to the

growth of *C. orellanus* mycelia than nitrate. The relatively limited growth on nitrate was responsible for the significantly higher total nitrogen and total phosphorus concentrations (per g of D.W.) in the mycelia grown on nitrate, compared to these grown on reduced nitrogen. The increase in total nitrogen on the Ni medium was essentially due to the accumulation of soluble organic nitrogen. As in other Homobasidiomycetes, the mycelium of *C. orellanus* accumulates only negligible quantities of nitrate (less than 0·05 % of the total nitrogen) and very small quantities of ammonium (0·1 % of the total nitrogen). Accumulation of soluble organic nitrogen was greater during growth on nitrate than on reduced nitrogen. Regardless of the medium, almost all of the soluble organic nitrogen was in the form of amines. The percentage of chitin nitrogen relative to total nitrogen was smaller in *C. orellanus* than in other Homobasidiomycetes (Mention & Plassard, 1983).

These results indicate behavioural differences related to nitrogen and phosphorus accumulation between *C. orellanus* and isolates of *H. cylindrosporum*. *P. tinctorius*, *S. granulatus* and *S. luteus* (Mention & Plassard, 1983; Mousain, unpubl.). In view of these observations, it will first be necessary to verify the hypothesis stated above (see Results) by assaying soluble nitrogenous molecules accumulated in the mycelia of *C. orellanus* cultured on nitrate or ammonium and to test the hypothesis by studying the enzymes involved in the nitrogen metabolism (particularly the activities of the enzymes that reduce nitrate and assimilate NH_3).

Lastly, to make the biosynthesis of orellanine feasible, it will be necessary to study the mechanisms that regulate its synthesis particularly with respect to the precursors of the synthesis. The production of this molecule containing two nitrogen atoms may depend on several factors in the medium, including the form of available nitrogen.

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