Nutritional Requirements for Growth of *Dispira cornuta* in Axenic Culture

By N. SINGH*

Department of Biological Sciences, University of Exeter, Exeter EX4 4PS

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INTRODUCTION

The nutritional requirements of haustorial mycoparasites remain uncertain, even though extensive axenic growth of certain mycoparasites has been obtained following the addition of a growth factor, mycotrophein, to an appropriate basal medium (Barnett & Lilly, 1958; Whaley & Barnett, 1963; Gain & Barnett, 1970; Calderone & Barnett, 1972). Limited growth of mycoparasites in the absence of host extracts has been reported for *Syncephalis* (Ellis, 1966) and *Dispira parvispora* (Brunk & Barnett, 1966). Barnett (1970) successfully cultured four haustorial mycoparasites including *Dispira cornuta* on a glycerol-casein hydrolysate medium, and Binder & Barnett (1974) reported excellent axenic growth of *Tieghemomyces parasiticus* on a glycerol-casein hydrolysate or amino acid medium.

There are different reports regarding the nutrition of *D. cornuta*. Ayers (1933) reported abundant axenic growth on natural media such as meat, fish and eggs. However, Kurtzman (1968) was unable to confirm Ayers' results. Out of five isolates of *D. cornuta* studied by Kurtzman only three made fair axenic growth on glycerol or acetate media in the presence of host extract, and these three isolates failed to utilize glucose. Using Kurtzman's isolate 1, Barker & Barnett (1973) reported that, in a medium containing glycerol, increasing amounts of casein hydrolysate, L-glutamic acid and L-aspartic acid supported correspondingly increasing amounts of growth, with an optimum at 40 g/l. L-Aspartic acid was the best nitrogen source. How such large amounts of L-aspartic acid and L-glutamic acid were kept in solution when the solubilities of these amino acids at 25 °C are 5 and 8.64 g/l respectively was not explained. It is therefore doubtful whether either of these amino acids was completely dissolved at the optimum concentration for the growth of the fungus (40 g/l).

To understand the physiology of this very specialized group of mycoparasites, the nutritional requirements of a large number of species and isolates need to be investigated. I report the results of a nutritional study of an isolate of *D. cornuta*.

METHODS

Organism. A culture of *Dispira cornuta* growing on *Cokeromyces recurvatus* originating from the Commonwealth Mycological Institute, Kew, Surrey (C.M.I. No. 77591) was obtained from the Botany Department, Birkbeck College, University of London.

Cultures of the mycoparasite free of its host were established by lifting the sporangio-phores of the parasite under a binocular microscope with a fine sterile needle and streaking these on solidified basal medium (see below). Care was taken to exclude the host sporangio-phores. After the spores of the mycoparasite had germinated, hyphal tips were transferred

* Present address: Department of Botany, Fourah Bay College, University of Sierra Leone, Freetown, Sierra Leone.

to fresh basal medium and the pure culture thus established was maintained on this medium.

Media and standard conditions. The basal medium (BM) consisted of: glucose, 20 g; L-asparagine, 2 g; K_2HPO_4 , 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; thiamine, 500 μ g; distilled deionized water to 1 l; buffered at pH 6.0 by 0.01 M-phosphate buffer. Concentrations of different carbon and nitrogen sources, where these were substituted for glucose or asparagine in BM, are specified below. Liquid medium (25 ml) was dispensed in 100 ml rimless conical flasks with Oxoid metal caps. Since shaking enhanced growth considerably, cultures were shaken on a reciprocal shaker at 24 ± 1 °C. Four replicates constituted a sample. For dry weight determinations, mycelium was filtered and washed through sintered glass crucibles and dried at 80 °C overnight.

Inoculum. Mycelium of the mycoparasite grown on liquid BM was blended for 1 min in a Waring blender and washed three times with sterile distilled water by centrifugation. The final pellet was resuspended in 20 vol. of sterile distilled water and 1 ml of this suspension was used to inoculate each flask.

Analysis. Glucose was determined by the method of Loewus (1952). Total nitrogen was converted to ammonia by a modification of the technique of Lang (1958), selenium oxy-chloride being replaced by an equivalent amount of selenium dioxide. In the conversion of amide of asparagine to ammonia and the distillation of ammonia, the method of Conway (1950) was followed. Ammonia determinations were done by the Nesslerization procedure of Yuen & Pollard (1952).

RESULTS

Effect of different carbon sources

Carbon sources were employed at concentrations which gave carbon equivalent to 2 % (w/v) glucose in the medium. The best growth occurred on starch. Glycerol, acetate and several sugars were poorly utilized (Table 1).

Effect of different nitrogen sources

Various nitrogen sources provided nitrogen equivalent to 0.25 % asparagine. There was good utilization of casein hydrolysate, urea, asparagine, aspartic acid, serine and arginine (Table 1). Glutamic acid, glycine and ammonium sulphate were intermediate sources of nitrogen, while alanine, cysteine, glutamine, leucine, phenylalanine and peptone were poorly utilized. Only slight growth occurred on histidine, lysine, methionine, proline, threonine, valine and potassium nitrate.

An increase in the rate of growth and total dry weight of mycelium, caused by increased concentrations of casein hydrolysate and two amino acids in the medium, has been reported by Barker & Barnett (1973) for another isolate of *D. cornuta*, although their results are doubtful (see introduction). We therefore investigated the effects of increased concentrations of asparagine and casein hydrolysate in the medium, with 2 % (w/v) glucose as carbon source. Increase in asparagine from 0.25 to 2 % (w/v) did not produce significantly higher yields of mycelium. However, final dry weight rose when the concentration of casein hydrolysate was increased above 0.37 % and was optimal at 1.48 % (equivalent to 1 %, w/v, asparagine in N content). At 2.96 % (w/v) casein hydrolysate (equivalent to 2 % asparagine in N content), there was a decline in growth which became very marked at 5.92 % casein hydrolysate (equivalent to 4 %, w/v, asparagine in N content).

To determine whether asparagine and casein hydrolysate act as carbon as well as nitrogen sources, the spent media from the above experiment were analysed for residual asparagine

Table 1. *Yield of D. cornuta on media with various carbon sources, nitrogen sources, and amendments*

Carbon source	Yield* (mg dry wt/ flask)	Final pH	Nitrogen source (<i>cont.</i>)	Yield* (mg dry wt/ flask)	Final pH
Glucose	78.0 ± 3.8	4.7	L-Leucine	25.0 ± 2.3	4.0
Fructose	68.3 ± 6.7	5.9	L-Lysine	3.5 ± 0.7	4.2
Rhamnose	6.0 ± 2.0	7.0	L-Methionine	8.0 ± 0.7	4.2
Galactose	8.8 ± 0.7	7.2	L-Proline	3.5 ± 1.8	4.2
Arabinose	2.3 ± 0.7	6.5	L-Phenylalanine	28.0 ± 2.7	4.1
Xylose	5.3 ± 3.5	6.4	DL-Serine	61.0 ± 2.9	3.9
Lactose	4.8 ± 2.5	6.5	Peptone	30.8 ± 0.7	4.4
Maltose	65.5 ± 3.2	5.1	L-Threonine	15.5 ± 1.8	4.0
Sucrose	4.0 ± 1.0	6.1	L-Valine	15.3 ± 1.8	3.9
Starch	96.3 ± 6.8	4.9	Casein hydrolysate	87.5 ± 3.5	4.3
Dextrin	87.8 ± 8.2	6.2	Potassium nitrate	14.3 ± 1.8	4.2
Glycerol	6.5 ± 2.4	7.1	Ammonium sulphate	48.8 ± 5.4	4.1
Potassium acetate	4.0 ± 2.0	8.2	Urea	90.3 ± 5.7	4.5
Nitrogen source			Amendment†		
L-Asparagine	78.5 ± 4.1	4.0	None (BM without thi)	26.3 ± 2.3	4.2
L-Aspartic acid	75.5 ± 3.4	4.2	BM + thi	71.8 ± 2.2	4.4
L-Arginine	63.0 ± 5.3	3.8	BM + o.v.	39.5 ± 3.4	4.2
L- α -Alanine	38.3 ± 2.9	3.9	BM + thi + o.v.	82.5 ± 7.5	4.6
L-Cystein	36.8 ± 4.6	3.9	BM + m.e.	31.5 ± 5.0	4.5
L-Glutamic acid	42.5 ± 3.1	4.5	BM + m.e. + thi	105.8 ± 4.3	4.6
Glutamine	27.3 ± 2.9	3.9	BM + m.e. + o.v.	44.8 ± 4.4	4.3
Glycine	42.8 ± 3.5	4.2	BM + m.e. + thi + o.v.	99.0 ± 4.9	4.3
L-Histidine	4.0 ± 0.7	3.8			

* Mean of four replicates, with standard errors.

† BM, basal medium. thi, Thiamine. o.v., Other vitamins, consisting of (μ g/l): thi, 500; biotin, 10; calcium pantothenate, 400; *p*-amino benzoic acid, 100; folic acid, 100; mesoinositol, 2000; pyridoxine, 400; riboflavin, 200; nicotinamide, 100. m.e., Microelements – to 1 l of BM were added 10 ml of a stock solution containing (mg/l distilled deionized water): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10; MnCl_2 , 1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 7.5.

and casein hydrolysate, production of ammonia and residual glucose. Unused nitrogenous compounds were still present in the media after 6 days of growth except at the lowest concentration. A considerable portion of the nitrogenous compounds decomposed remained in the medium as ammonia, and media with higher concentrations of asparagine and casein hydrolysate showed correspondingly higher concentrations of ammonia. In another experiment, analysis of spent medium showed that the fungus did not utilize asparagine carbon even when the glucose concentration in the medium was reduced to 0.05 %.

Effect of vitamins and microelements

To study the effects of vitamins and trace elements, BM was used with various amendments. Thiamine stimulated growth, while other vitamins had little or no effect. Microelements promoted growth in the presence of thiamine but not in the presence of other vitamins (Table 1).

Shigo, Anderson & Barnett (1961) reported that an increase in the concentration of manganese in the medium was associated with an increase in the degree of parasitism of *Piptocephalis xenophila*. In the present study, however, additions to the basal medium of microelements and manganese alone at concentrations four times higher than those used in the previous experiment did not enhance growth significantly.

DISCUSSION

The utilization of glycerol and failure to utilize glucose and other sugars, first discovered by Kurtzman (1968), was emphasized as the key to the successful axenic culturing of *D. cornuta* and other haustorial mycoparasites by Barnett (1970). However, in the present study glycerol and acetate were not utilized, whereas glucose and other sugars were. Starch was the best carbon source but it is possible that it was partially hydrolysed during autoclaving and it was the products thus released which were responsible for the good growth. The good growth on glucose, fructose and maltose cannot be related to the reducing nature of these sugars, since galactose, another reducing sugar, was not utilized well. This isolate used a wider range of nitrogen sources than that reported by Barker & Barnett (1973) for their isolate. Ammonium sulphate, reported by Barker & Barnett (1973) to be a poor source of nitrogen, was a reasonably good one. The poor utilization of nitrate is in agreement with the results obtained by Kurtzman (1968). The utilization of amino acids was not found to be related to their structure. For example, alanine was a good source of nitrogen, while histidine and lysine, belonging to the same group (basic), were not utilized. Optimal growth was obtained in media containing 1 % asparagine or 1.48 % (w/v) casein hydrolysate. There was a decline in growth at higher concentrations, probably because of toxicity caused by ammonia which builds up in cultures as the concentrations of nitrogen sources are increased. This could also explain the rise in final pH of cultures with increasing initial concentrations of nitrogen. These results are in contrast to those obtained by Barker & Barnett (1973), who reported optimum growth of their isolate of *D. cornuta* with 40 g casein hydrolysate, L-aspartic acid or L-glutamic acid/l. The fungus had a deficiency for thiamine, but not for any other vitamin. Barker & Barnett (1973) reported that their isolate had a clear deficiency for both thiamine and biotin. Some growth on basal medium without vitamins was probably due to the contamination of other constituents of the medium with vitamins. Microelements were stimulatory only in the presence of thiamine, and this stimulation was not due to manganese alone (Shigo *et al.* 1961).

This isolate of *D. cornuta* did not show the same nutritional pattern as other isolates of *D. cornuta* and other haustorial mycoparasites so far studied in axenic culture.

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