Nutritional Studies of *Zygorhynchus* Species

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SUMMARY: Nutritional experiments were carried out dealing with: (1) the utilization of carbohydrates; (2) the nitrogen source; (3) the action of different mineral salts and their interaction. The results showed that the strains of *Zygorhynchus* used grew well at 25° at pH 8.0 when buffered with nutritionally inert tris buffer and incubated for 12-14 days. Statistical analysis of the results showed that under the given conditions optimal growth measured as mg. dry wt. was obtained in liquid media containing 4% (w/v) glucose; 0.1 M tris buffer; 0.00625 M-(NH₄)₂SO₄; 2 p.p.m. Fe, Zn and Mn; with the following optimal conditions for the different strains specified: (i) for *Z. moelleri* CMI 0.002 M-KH₂PO₄; 0.012 M-MgSO₄.7H₂O; (ii) for *Z. moelleri* Baarn 0.002 M-KH₂PO₄; 0.006 M-MgSO₄.7H₂O; (iii) for *Z. vuillemini* CMI 0.002 M-KH₂PO₄; 0.008 M-MgSO₄.7H₂O; (iv) for *Z. vuillemini* Baarn 0.004 M-MgSO₄.7H₂O. There was a balance between the possible pairs of the salts from KH₂PO₄, MgSO₄.7H₂O and (NH₄)₂SO₄ (in one case only between KH₂PO₄ and MgSO₄.7H₂O). There was an optimal balance between all the salts for two strains. The best carbon sources were glucose, fructose, mannose, xylose and arabinose. The best sources of nitrogen were L-glutamine, L-asparagine, glycine, (NH₄)₂SO₄.

In 1953 Burges & Fenton observed that in the acid soils of East Anglia *Zygorhynchus vuillemini* was abundant below 10 cm., but relatively scarce in the surface regions; this distribution was correlated with a high tolerance to carbon dioxide. Several workers have studied the effect of different media on the reproductive processes of *Zygorhynchus* spp. but no detailed account of the general mineral nutrition of members of this genus has been given. The purpose of the present paper is to report on a nutritional study of some different *Zygorhynchus* spp.

METHODS

The following organisms were used. *Zygorhynchus moelleri* and *Z. vuillemini* from the Commonwealth Mycological Institute (CMI) which are zygospor-producing strains; *Z. moelleri* and *Z. vuillemini* from the Central Bureau voor Schimmelcultuur, Baarn (Baarn), which were non-zygospor-producing strains. Throughout this paper the following abbreviations are used: CMI strains are designated MC and VC, respectively, while the Baarn strains are designated MB and VB, respectively.

Single spore cultures of these fungi were kept as stocks in 6 in. × $\frac{3}{4}$ in. test tubes on potato glucose agar at 0° and subcultured as required. No alteration of morphological and cultural characteristics of these fungi occurred during the

period of the experiments. Exploratory experiments with VC showed that in phosphate-containing media a good source of inorganic nitrogen was $(\text{NH}_4)_2\text{SO}_4$, but the use of this substance led to an accumulation of H_2SO_4 in the medium (see also Machlis, 1953; Fothergill & Yeoman, 1956), to a consequent decline in pH value, and to the phosphate acting as a buffer as well as a nutrient. The use of an inert buffering agent was thus desirable. Tris buffer (2-amino-2-hydroxymethylpropane-1:3-diol) was found to be suitable.

In the nutritional experiments inoculations were made into a final volume of 25 ml. sterile culture fluid in Pyrex Erlenmeyer flasks (150 ml.) and incubated at 25°. Sterile phosphate solution was added aseptically from a glass syringe after autoclaving in order to avoid precipitation. The inoculum was a suspension of spores and mycelial fragments made by adding 10 ml. sterile distilled water to a suitable agar slope, shaking and decanting the liquid; 1 ml. of which was then added to each culture flask of liquid medium. It was found that the exact amount of the inoculum did not appreciably affect the amount of growth within the limits of the experiments.

All experiments were carried out on five replicates unless otherwise stated. After the growth period the mycelia were filtered on to tared Whatman No. 5 filter-papers and washed with distilled water. The mycelium with the filter-papers was then dried overnight at 85°, cooled in a desiccator and weighed; results are expressed as mg. dry wt. mycelium/flask. It was not found practical to separate mycelium from filter-paper after filtration, and as these filter-papers lose 7% of their original weight on drying under the above conditions, appropriate allowance was made for this. For preliminary experiments the following basal defined liquid medium A was used: 4% (w/v) glucose; 0.004 M- KH_2PO_4 ; 0.002 M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0125 M- $(\text{NH}_4)_2\text{SO}_4$; Zn, Fe and Mn at 2 p.p.m.

When tris buffer to 0.1 M was used the mineral constituents had to be modified for maximum growth in all cases to contain 0.002 M- KH_2PO_4 ; 0.006 M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; and 0.00625 M- $(\text{NH}_4)_2\text{SO}_4$; the complete modified medium containing tris buffer will be referred to as medium B. Preliminary experiments indicated that the temperature for maximum mycelial yield was 25°; a decrease in dry weight began on the 12th day of incubation with organisms MC and VC, and on the 14th day for organisms MB and VB.

RESULTS

Carbon requirements

The utilization of different carbon compounds by the strains of *Zygorhynchus* used was determined after 7-, 14- and 28-day growing periods. The basal medium B was used, but glucose was replaced by other carbon compounds at 15 g./l. Caramelization of the sugars was largely avoided by careful autoclaving, although this usually occurred to a small degree. The pH value of the resultant solutions after the addition of carbohydrates was adjusted to 8.0 which is a favourable value for these fungi. The following carbohydrates were used in this experiment, glucose, fructose, mannose, galactose, sorbose,

arabinose, xylose, maltose, lactose, sucrose and raffinose. The results showed that there was a large variation among the organisms tested in their ability to use, and their rate of utilization of, the different carbohydrates. After 7 days of incubation of organism MC only glucose, fructose and mannose were utilized to any large extent, but after 28 days yields of about 170 mg. dry wt. mycelium were obtained, and also with galactose or mannose; yields of mycelium with xylose and arabinose were also high. A similar pattern of utilization obtained also for organism MB but with a smaller yield of mycelium. With sucrose organism MB produced 141 mg. dry wt. after 28 days as against 24 mg. for organism MC. Incubation of organisms MC and MB for 7 days gave relatively small yields with all carbohydrates, but after 14 and 28 days all except sorbose, lactose and raffinose were utilized. The Baarn strains, unlike the CMI strains, utilized sucrose (125 mg. dry wt. mycelium as against 36 mg.). Maltose gave only 41 mg. dry wt. of mycelium with organism MB after 14 days but 182 mg. after 28 days incubation; organism MC gave 161 mg. dry wt. mycelium with arabinose, while organism MB gave only 35 mg. In general, both of the CMI strains gave consistently higher weights of mycelium than the Baarn strains.

Lilly & Barnett (1953) made a comprehensive study of the effect of sugars on the growth of a large number of fungi which included *Zygorhynchus vuillemini*. Their basal medium, however, differed from that used here and the pH value was considerably lower. A comparison is therefore not strictly accurate, but nevertheless may be made. In both sets of experiments glucose, fructose, mannose and galactose were good sources of carbon, while sorbose and lactose were the poorest sources. Organism VC was again exceptional as giving a high yield with arabinose.

Nitrogen requirements

In this experiment the effect of different sources of nitrogen was determined. The basal medium B was used with glucose and the nitrogen salt was varied but supplied at a nitrogen concentration equivalent to 0.00625M- $(\text{NH}_4)_2\text{SO}_4$. The pH value was adjusted to 8.0 in all cases. With all strains used there was a steady increase in nitrogen utilization in all its forms tested during 7–14 days of incubation; at 28 days there was less dry wt. of mycelium than at 14 days. In all strains the highest yields of mycelium were obtained with L-glutamic acid, L-asparagine or glycine (from 182 to 236 mg. dry wt. mycelium); $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 gave the next highest average yields (156–163 mg. dry wt. mycelium); while aspartic acid gave 121 mg. and KNO_3 only 76 mg. In general, as with the carbon requirement, the Baarn strains gave consistently lower yields of mycelium than those from the CMI. Thus these fungi fall into Robbins's (1937) second class according to their ability to utilize mineral nitrates, ammonium salts and organic forms of nitrogen.

Factorial experiments

The object of these experiments was to determine the effect of varying the concentration of the mineral salts and ammonium sulphate in the basal

medium. The factorial design and statistical analysis of the results should indicate whether or not a balance between the salts is necessary for high mycelial yields under the given conditions. The direct effect of the individual salts in the medium and the interaction between them is also determined. Medium B was used in these experiments; cultures were incubated at 25° for 12 days for CMI strains and for 14 days for Baarn strains. The results were expressed as mg. dry wt. mycelium average of four replicates in each case. The concentrations of the salts were fixed on the basis of halving and doubling them in the basal medium. Thus, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{SO}_4$ were each used at three concentrations and all possible combinations of them were set up. In any one experiment with any one strain 108 culture flasks were incubated. The initial pH value of all solutions was 7.8–8.0 and any tendency for a decrease in pH value was controlled by the tris buffer. The results are shown in Tables 1 and 2 and the analyses of variance in Table 3.

Table 1. *Growth responses of four strains of Zygorhynchus moelleri and Z. vuillemini to varying concentrations of KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{SO}_4$*

Concentration of KH_2PO_4 : P_1 , P_2 , $\text{P}_3 = 0.001\text{M}$, 0.002M , 0.004M ; of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: Mg_1 , Mg_2 , $\text{Mg}_3 = 0.008\text{M}$, 0.006M , 0.012M ; of $(\text{NH}_4)_2\text{SO}_4$: N_1 , N_2 , $\text{N}_3 = 0.008125\text{M}$, 0.00625M , 0.0124M .

A. *Z. moelleri* CMI (=MC) and *Z. moelleri* Baarn (=MB)

	P_1						P_2						P_3					
	Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3	
	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB
	Mean dry wt. mycelium (mg./flask)																	
N_1	123	117	125	123	132	120	134	118	135	118	136	117	130	116	131	119	143	127
N_2	164	152	169	155	176	153	175	168	178	170	183	162	143	171	166	165	182	166
N_3	147	154	160	155	165	155	140	148	144	145	161	144	125	147	127	142	184	142

B. *Z. vuillemini* CMI (=VC) and *Z. vuillemini* Baarn (=VB)

	P_1						P_2						P_3					
	Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3	
	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB
	Mean dry wt. mycelium (mg./flask)																	
N_1	142	128	134	122	128	121	150	135	145	122	143	122	144	136	141	124	140	119
N_2	144	136	141	137	141	136	151	132	151	137	151	130	147	132	144	139	145	130
N_3	139	125	139	122	139	119	145	125	149	123	142	119	141	137	142	123	142	120

With organism MC there were significant differences between the yields and all concentrations of salts used except between 0.001M and 0.002M - KH_2PO_4 . The highest yield was produced with P_2 , Mg_3 and N_2 , which gave 183 mg. dry wt. mycelium. With organism MB there was no significant difference in yields with any of the concentrations of either KH_2PO_4 or MgSO_4 . Ammonium sulphate at 0.00625M gave 163 mg. dry wt. mycelium. The best growth was obtained with combination P_2 , Mg_2 and N_2 . With organism VC, except for the 0.002M concentration where the increase of dry wt. was 10 mg., any

increases in dry weights were small but significant. The highest weight of mycelium produced was with P_2 , Mg_1 and N_2 . With organism VB all concentrations of $MgSO_4$ and $(NH_4)_2SO_4$ produced significant differences of yield, but with KH_2PO_4 the variation in yield was not large. The highest yield of mycelium with organism VB was produced with P_3 , Mg_1 and N_2 . With all strains 0.00625 M- $(NH_4)_2SO_4$ gave the highest yields. The analyses of variance show that all first-order interactions were significant except that between KH_2PO_4 and $MgSO_4$; with organism VC that between $(NH_4)_2SO_4$ and $MgSO_4$ was significant only at the lower 19:1 odds. It is evident that with all four strains of *Zygorhynchus* used the concentration of ammonium sulphate and the interaction of this with KH_2PO_4 were important in order to obtain high yields of mycelium. In the second-order interactions between the three salts taken together only organism MC showed a high interaction at the 99:1 level of significance and organism MB at the 19:1 level.

Table 2. Mean dry weights and growth responses of *Zygorhynchus* spp. after 12 and 14 days incubation at 25° on media with different concentrations of certain components

Concentrations of salts as in Table 1, $KH_2PO_4 = P_1, P_2, P_3$; $MgSO_4 \cdot 7H_2O = Mg_1, Mg_2, Mg_3$; $(NH_4)_2SO_4 = N_1, N_2, N_3$. Difference required between weights for odds of 99:1 for strain MC = 2.78; for strain MB = 2.34; for strain VC = 2.08; for strain VB = 2.04.

Concn.	Organisms			
	MC	MB	VC	VB
	Mean dry weight mycelium (mg./flask)			
P_1	152	143	137	127
P_2	154	143	147	127
P_3	143	144	143	129
Mg_1	142	143	145	132
Mg_2	148	143	144	128
Mg_3	158	143	141	124
N_1	132	119	141	126
N_2	171	163	144	134
N_3	145	148	142	124
Mean	149.4	143.2	142.5	127.8

General mean = 140.7 mg. dry wt. mycelium/flask.

In these factorial experiments KH_2PO_4 acted as a nutrient because its buffering effect was counteracted by the tris buffer which maintains the acidity at a constant value (Fothergill & Yeoman, 1956). The experiments indicated that a high degree of balance between the salts of the first order of interaction produced the highest yields of mycelium, but this balance was only absolute between all the salts in the second order of interaction in the case of organism MC and to a less extent of organism MB. Variation of balance in salt concentrations was thus shown between two different species of the same genus, i.e. between *Z. moelleri* and *Z. vuillemini*. In previous experiments of similar factorial design Talley & Blank (1941) and Fothergill & Ashcroft (1955) showed that a correct balance between the mineral constituents of the

medium was essential in order to obtain a high yield of mycelium with two parasitic fungi, *Phymatotrichum omnivorum* and *Venturia inaequalis*, respectively. Fothergill & Raine (1954) showed that while a correct balance between salts was conducive to good growth, it was not of overriding importance with complementary strains of the saprophytic fungus *Mucor hiemalis*. Fothergill & Yeoman (1957) found with the saprophyte *Rhizopus stolonifer* that a balance existed between K_2HPO_4 and $(NH_4)_2SO_4$, but not between these and the other salts in the medium.

Table 3. *Analysis of variance (grouped) for Zygorhynchus strains MC, MB, VC and VB*

Required 'F' and 't' values taken from Snedecor's tables (1934). Sums of squares and mean squares are omitted from table.

Variance	D.F.	F required odds					
		Found				99:1	19:1
		Organism					
		MC	MB	VC	VB		
Total	107	—	—	—	—	—	—
$(NH_4)_2SO_4$	2	757.15	1317	28.3	108.5	4.88	3.11
KH_2PO_4	2	65.157	0.41	11.6	4.7	4.88	3.11
$MgSO_4 \cdot 7H_2O$	2	111.667	—	7.07	679.5	4.88	3.11
$(NH_4)_2SO_4 \times KH_2PO_4$	4	58.071	39.4	7.1	5.1	3.56	2.49
$(NH_4)_2SO_4 \times MgSO_4$	4	6.847	3.749	2.62	23.5	3.56	2.49
$KH_2PO_4 \times MgSO_4$	4	7.803	4.497	1.68	3.6	3.56	2.49
$KH_2PO_4 \times MgSO_4 \times (NH_4)_2SO_4$	8	10.595	2.612	1.1	1.3	2.74	2.06
Residual error	81	—	—	—	—	—	—

The strains of *Zygorhynchus* used in the present experiments show several major differences in their nutritional requirements and growth rates. The onset of decrease of dry wt. of mycelium varies with strain from 12 days with the non-sporing forms to 14 days in the sporing forms. *Zygorhynchus moelleri* and *Z. vuillemini* from the CMI were unable to utilize sucrose as source of carbon, while the strains from Baarn were able to use it to a marked extent. This may indicate the lack of invertase in the British strains. The Baarn strain of *Z. vuillemini* utilized arabinose, but all the strains used sorbose, lactose or raffinose to a small extent, or not at all. Lilly & Barnett (1953) found that sorbose had an inhibitory effect on the growth of several fungi. The hydrolytic products of lactose and raffinose were utilized by all the strains of *Zygorhynchus* tested. Thus it seems that these fungi lack the enzymes necessary for the hydrolysis of the higher sugars.

In the experiment on the nitrogen requirement only *Zygorhynchus moelleri* CMI used KNO_3 to any marked extent. The sporing strains of *Zygorhynchus* gave low mycelial yields with L-glutamine while the non-sporing strains gave high yields. The sporing strains also required higher Mg concentrations than the non-sporing strains; this may be correlated with Hawker's observation (1950) that magnesium concentration influences sporulation. Hence it may be

concluded that whilst these four strains of *Zygorhynchus* have similar basal nutritional requirements there are important differences between them.

The writers thank their colleagues Dr D. A. Evans, and Mr H. Campbell for their assistance with the statistical part of the paper.

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(Received 14 March 1958)