***Aspergillus niger* Technique for the Bioassay of Manganese**

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(Accepted for publication 3 August 1967)

SUMMARY

The rates of growth of five strains of *Aspergillus niger* (M, MUBLI, NRRL 334, NRRL 323, NRRL 346) were followed in an optimum nutrient solution. The time course of growth of all the strains showed a steep decrease after 3 days, except that of strain NRRL 346 which tended to flatten out. A correlation between sporulation and mycelial dry weight was observed. All the five strains showed a marked decrease in mycelial yield in the absence of manganese from the basal medium. Their quantitative responses to graded concentrations of Mn in the medium were compared with spore and mycelial inocula. Statistical analyses of the result suggest that strain MUBLI is the most suitable test organism for the bioassay of Mn. The effective range of concentrations of Mn which coincided with the maximum differences in mycelial dry weights was 0.001–0.006 µg./ml.

INTRODUCTION

Many attempts have been made in the past two decades to study the need for trace elements for the growth of micro-organisms in general and fungi in particular. The use of fungi as test organisms for the assay of trace metals has come into vogue because of their specific response to deficiency of individual trace metals in the nutrient medium. Several strains of *Aspergillus niger* have been used in the assay of trace elements under various physical and physiological conditions (Mulder, 1948; Nicholas & Fielding, 1951; Nicholas, 1952; Donald, Passey & Swaby, 1952*a*; Saraswathi-Devi, 1962). The lack of uniformity in experimental conditions has made comparison of the behaviour of various strains to deficiencies of trace metals impossible. Furthermore, since purification of nutrient solutions is an essential prerequisite for obtaining precise deficiency symptoms, attention has been mainly devoted to the methods of purification and little recognition has been given to the fact that comparison between the quantitative response of organisms under physiologically indeterminate conditions would not be of great value. The need for a balanced optimum solution for growth, containing a minimum quantity of constituents and with a composition subject to exact definition on the basis of physiological response was emphasized by Steinberg & Bowling (1939). Such a nutrient solution provides an accurate basis for the comparison of the quantitative response of different strains of the same organism within a defined physical environment. An optimum nutrient solution has been used in the present work to compare the response of five strains of *A. niger* to a graded range of concentrations of manganese in the nutrient solution with a view to selecting a strain for the bioassay of this element.

Bioassay methods are in general based on the observation that in the absence of an essential mineral element growth is retarded as shown by a decrease in mycelial dry

weight. Bioassay of manganese has so far been done by visual comparison of mycelial characters and spore cover between the standards and the unknown since decrease in growth in the absence of manganese could not be obtained (Nicholas, 1952) although Steinberg (1939) reported clear-cut decrease in growth of *Aspergillus niger* in manganese-deficient medium. An attempt was made in the present work to enquire whether it is possible to obtain decreases in mycelial yields in manganese-deficient cultures in an otherwise optimum nutrient solution, by using the purification procedure of Donald *et al.* (1952*b*), and whether the growth of the organism is reproducibly and quantitatively proportional to graded increments in the concentration of manganese. When decrease in growth is considered as the criterion for examining the need for a micro-nutrient element, a knowledge of the rate of growth and the relationship between growth and reproduction is necessary as comparisons are best made during the phase of maximal growth. Hence the rates of growth of the five strains of *A. niger* were also studied.

METHODS

The following strains of *Aspergillus niger* were used:

M, Mulder's strain—culture received from Centraal Bureau voor Schimmelcultures, Baarn, Holland.

MUBL I, isolated from soil at the University Botany Laboratory, Madras and used previously in this Laboratory for micronutrient element deficiency studies.

NRRL 334, the original no. Thom. 4247 used by Steinberg in his studies on physiology of *Aspergillus niger* with special reference to heavy metal nutrition.

NRRL 323, used for soil testing by Niklas & Hirschberger (1924).

NRRL 346, characterized by abundant production of sclerotia.

The basal medium contained (g./l.): ammonium nitrate, 2.06; monopotassium phosphate, 0.55; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; sucrose, 50 (Steinberg, 1939). The trace element composition of the medium was as follows (mg./l.): Fe, 0.20; Zn, 0.20; Cu, 0.04; Mn, 0.02; Mo, 0.01. The basal medium was made up with double-distilled water and was initially purified from trace elements by adsorption with aluminium oxide according to the method of Donald *et al.* (1952*b*). The medium at pH 7.2 with 0.5% (w/v) Al_2O_3 (chromatographic analysis grade) was autoclaved at 115° for 20 min. The medium was swirled while still hot and then allowed to stand overnight; it was then filtered through Whatman no. 42 by gravity filtration. The filtered medium was then adjusted to pH 7.5 and distributed into 100 ml. Erlenmeyer flasks; each flask had 15 ml. medium containing the appropriate concentration of trace elements. The trace element stock solutions and dilutions were made in triple-distilled water. The 'minus all' trace-elements medium was the purified basal medium without any added trace element, the 'plus-all' medium contained all the trace elements, and the 'minus Mn' medium had all the trace elements except manganese. The media were autoclaved at 121° for 15 min. The chemicals used were of the Analar or Merck G.R. grade and water was distilled in all-glass Pyrex stills. All the precautions which were essential for the *Aspergillus niger* technique (Saraswathi-Devi, 1958) were observed.

Spore inoculum was prepared by shaking 3 loops of spores from 5-day cultures on a potato glucose agar slope into 5 ml. of sterile triple-distilled water; each flask received three drops of this spore suspension. Mycelial inoculum was prepared by growing the

fungus in 'minus all' medium for 3 days. The mycelium was then filtered, washed and broken with Pyrex glass beads in 10 ml. of sterile distilled water; three drops of this suspension were added to each flask. The flasks were randomized and incubated at $35 \pm 0.2^\circ$. The cultures were filtered at appropriate times and the mats dried at 80° to constant weight.

Since only three replicates could be maintained for each concentration of Mn in one experiment, the above experiments were repeated and essentially similar results were obtained.

The rate of growth was measured by recording the mycelial dry weight at 24 hr intervals for 6 days. Statistical analyses were done according to Paterson (1939) and the regression analysis was done according to Bailey (1961) and Freeman (1956). The best-fitting straight line $yr = a + bx$ was constructed by the method of least squares, where b is known as the regression coefficient of y on x . The test of significance used was the conventional F test at 5 % and 1 %.

RESULTS

The time course of growth of all the five strains was characterized by three distinct phases: (1) a phase of little or no apparent growth extending for 24 h after inoculation; (2) a phase of rapid and approximately linear growth between 24 and 72 h; (3) a phase of no net growth or of decrease in dry weight after 72 h (Fig. 1). This is typical of many filamentous fungi grown in liquid culture (Cochrane, 1958). Sporulation started

Table 1. *Rate of growth of five strains of Aspergillus niger*

Incubation (days)	Strain				
	NRRL 346	NRRL 334	NRRL 323	MUBL I	M
	Mycelial dry weight (mg.)				
1	7 ± 6*	11 ± 5	18 ± 7	13 ± 7	2.4 ± 0.3
2	203 ± 14	258 ± 10	241 ± 2	215 ± 11	83 ± 3
3	291 ± 5	301 ± 2	313 ± 2	344 ± 13	292 ± 2
4	291 ± 5	283 ± 2	269 ± 4	318 ± 10	240 ± 4
5	284 ± 0.3	252 ± 4	246 ± 2	279 ± 19	239 ± 4
6	283 ± 3	242 ± 3	221 ± 2	223 ± 3	186 ± 2

* Standard error of mean.

on the 3rd or 4th day, i.e. at the end of the phase of rapid growth, with the exception of strain NRRL 346, which began to sporulate on the second day. Whether the decrease in dry weight of the mycelium after 72 hr was due to sporulation or to extensive autolysis of the mycelium is not clear. There appeared to be a correlation between sporulation and decrease in mycelial weight, the reproductive phase being presumably stimulated either by exhaustion of the nutrients in the medium or by autolytic products.

Statistical analysis of the data (Table 1) indicated that the mycelial dry weights at the end of 3 days of incubation was significantly greater than on the preceding or the following day. In some of the experiments, however, growth was not significantly different between 3 and 4 days, though the average weight of mycelium at the end of 4 days was always lower. Strain NRRL 346 was an exception in that there was no significant decrease in mycelial weight after reaching the maximum at the end of 3 days.

Response of two strains of Aspergillus niger to Mn deficiency

Two drops of spore suspension of the two strains MUBL I and M were added to 'minus all', 'minus Mn' and 'plus all' media and the flasks incubated at $35 \pm 0.2^\circ$. Each treatment had three replicates. One set of flasks were filtered on the 3rd day and another on the 6th day after inoculation; the results are presented in Table 2.

Both the strains showed a considerable decrease in mycelial yields in the absence of manganese, strain MUBL I showing lesser growth than strain M. In Mn-deficient media both strains produced discontinuous mycelia and there was no sporulation in these Mn-deficient cultures even at the end of 6 days.

Table 2. *Mycelial yield of two strains of Aspergillus niger in Mn-deficient cultures at two different incubation periods*

Strains	3 days			6 days		
	Minus all	Minus Mn	Plus all	Minus all	Minus Mn	Plus all
MUBL I	5.8	60.3	306.3	6.3	75.1	225.4
M	4.9	96.0	261.1	8.5	139.7	188.6

Table 3. *Effect of concentration of Mn on the growth of three strains Aspergillus niger*

Concentration of Mn ($\mu\text{g./ml.}$)	Spore inoculum			Mycelial inoculum		
	NRRL 346	NRRL 334	M	NRRL 346	NRRL 334	M
	Mycelial weight (mg.)					
1. 0.000	146	150	89	132	87	59
2. 0.001	216	211	212	204	110	88
3. 0.002	229	202	252	206	165	115
4. 0.004	259	273	271	237	222	162
5. 0.006	275	310	273	255	257	177
6. 0.008	292	303	259	265	256	185
7. 0.01	291	307	269	263	277	187
8. 0.02 ('plus all')	298	301	283	266	277	214
Basal medium ('minus all') alone	5	9	7	11	8	5

Statistical significance by *F* test at 5 %

Strain	Inoculum:spore suspension	Inoculum:mycelial suspension
M	1, 2, 3, 6, 7, 4, 5, 8*	1, 2, 3, 4, 5, 6, 7, 8
NRRL 346	1, 2, 3, 4, 5, 7, 6, 8	1, 2, 3, 4, 5, 7, 6, 8
NRRL 334	1, 3, 2, 4, 8, 6, 7, 5	1, 2, 3, 4, 6, 5, 7, 8

* The difference between treatments falling under the same bar are not significant.

Response of five strains of Aspergillus niger to concentration of Mn

The effect of graded quantities of Mn on the growth and sporulation of the five strains was studied under the conditions mentioned above. The required micro-nutrient elements were added, varying only the concentration of manganese. The Mn concentrations were 0.001, 0.002, 0.004, 0.006, 0.008, 0.01 and 0.02 $\mu\text{g./ml.}$ Spore and

Fig. 1

Fig. 2

Fig. 1. The rate of growth of five strains of *Aspergillus niger* in static cultures.

Fig. 2. Regression graphs showing the growth response of *Aspergillus niger* strains MUBL 1, NRRL 323 and NRRL 334 to graded increments in Mn concentration.

Table 4. Effect of concentration of Mn on the growth of three strains of *Aspergillus niger*

Concentration of Mn (μg./ml.)	Spore inoculum			Mycelial inoculum		
	NRRL 323	MUBL 1	M	NRRL 323	MUBL 1	M
Mycelial dry weight (mg.)						
1. 0.0	140	87	153	150	89	173
2. 0.001	183	177	252	220	151	264
3. 0.002	266	264	278	263	276	324
4. 0.004	300	345	295	293	355	336
5. 0.006	311	371	307	299	385	337
6. 0.008	314	375	292	300	381	344
7. 0.01	309	381	303	302	386	338
8. 0.02 ('plus all')	309	387	310	288	375	344
Basal medium (‘minus all’) alone	10	14	10	6	5	4

Statistical significance by *F* test at 5 %

Strain	Inoculum: spore suspension	Inoculum: mycelial suspension
M	1, 2, 3, 6, 4, 7, 5, 8	1, 2, 3, 4, 5, 7, 6, 8
NRRL 323	1, 2, 3, 4, 7, 8, 5, 6	1, 2, 3, 8, 4, 5, 6, 7
MUBL 1	1, 2, 3, 4, 5, 6, 7, 8	1, 2, 3, 4, 8, 6, 5, 7

mycelial inocula were separately used. In all these experiments the mycelial mats were harvested at the end of 3 days of incubation when maximum mycelial yield was obtained. The effect of Mn on sporulation was observed in separate sets of flasks which

were incubated for 6 days. Three strains were compared in each experiment, using either spore or mycelial inocula. The combination of strains used were as follows: (1) M, NRRL 346 and NRRL 334, (2) M, NRRL 323 and MUBLI. All strains showed a gradual increase in mycelial production with increasing concentration of manganese (Tables 3, 4). Statistical analyses indicated that strain M does not show significant variation in mycelial dry weight with variation in Mn concentration. In a few experiments, however, there was a significant increase in dry weight with 0.001 and 0.002 $\mu\text{g./ml.}$ Strain NRRL 346 showed difference in weight only between 'minus Mn' and 0.001 $\mu\text{g. Mn/ml.}$ and between 0.002 and 0.004 $\mu\text{g. Mn/ml.}$ Strain NRRL 334 was similar to strain NRRL 346 in its response when spore inoculum was used; however, significant differences between various levels of Mn were obtained with mycelial inoculum. Strains NRRL 323 and MUBLI showed significant increase in mycelial dry weight with increasing Mn concentration up to 0.004 and 0.006 $\mu\text{g./ml.}$, respectively, when spore inocula were used; mycelial inocula did not improve the response of these two strains.

Table 5. *Effect of concentration of Mn on the growth of five strains of Aspergillus niger (mycelial inoculum)*

Concentration of Mn ($\mu\text{g./ml.}$)	Strain				
	NRRL 346	NRRL 334	NRRL 323	MUBLI	M
	Mycelial dry weight (mg.)				
1. 0.0000	171	82	94	66	77
2. 0.0005	152	125	101	78	78
3. 0.001	197	133	127	91	102
4. 0.002	219	187	185	118	164
5. 0.004	244	243	250	236	157
6. 0.006	249	263	265	279	167
7. 0.02 ('plus all')	272	288	294	348	191
Basal medium ('minus all') alone	10	8	13	15	9

Statistical significance by *F* test at 5 %

NRRL 346	Not significant
NRRL 334	A, B, C, D, E, F, G
NRRL 323	A, B, C, D, E, F, G
MUBLI	A, B, C, D, E, F, G
M	A, B, C, D, E, F, G

Two features were observed in the above experiments: (1) there was no marked differences in the mycelial dry weight of any of the strains tested above 0.006 $\mu\text{g. Mn/ml.}$; (2) in the majority of the experiments there was a significant difference between 'minus Mn' and 0.001 $\mu\text{g. Mn/ml.}$ Hence the response of all the strains was compared for the following manganese concentrations: 0.0005, 0.001, 0.002, 0.004, 0.006 and 0.02 $\mu\text{g./ml.}$ The data presented in Table 5 confirmed the earlier observations on the response of all the strains. In addition, there was no significant difference between the growth obtained at 0.0005 $\mu\text{g. Mn/ml.}$ and 'minus Mn' cultures of strain M, MUBLI, and NRRL 323. Strain NRRL 334 showed no difference in growth between 0.0005 and 0.001 $\mu\text{g. Mn/ml.}$ On statistical analysis, the relationship

between concentration of Mn and growth exhibited by MUBLI and NRRL323 was found to follow a linear regression (Fig. 2) that is real and highly significant at both 5 % and 1 % levels, the observed F value being 161.34 and 43.1 respectively (The Table value of F at 5 % is 7.71 and at 1 %, 21.2) with degrees of freedom 1 and 4. For strain NRRL334, the linear regression is just significant at 5 % level only, the observed value of F being 8.05.

No attempt was made in the present work at a quantitative evaluation of sporulation in relation to Mn concentration. Visual observation indicated that sporulation started only at the concentration of 0.006 μg . Mn/ml. and that there was a good gradation in degree of sporulation from 0.006 to 0.02 μg . Mn/ml. for all the strains.

DISCUSSION

A study of the growth curves of the five strains of *Aspergillus niger* used in relation to sporulation suggests a correlation between growth and reproduction. Sporulation started towards the end of the phase of rapid growth in all strains except in NRRL346 and the increase in sporulation was accompanied by a decrease in dry weight of mycelium. Few studies have considered critically the relation of reproduction to growth phases. The data of Robinson (1926) for *Sporodinia grandis* Link indicated that in static culture, spores usually developed at the end of the phase of rapid growth; although spores may appear earlier (Cochrane, 1958).

In the absence of manganese, *Aspergillus niger* strain M showed a reduction in mycelial yields of more than 60–70 % of that in 'plus all' cultures; MUBLI showed a decrease of more than 75 %, whereas the other three strains showed only a 50 % decrease in growth. Nicholas & Fielding (1951) and Nicholas (1952) did not obtain decreases in mycelial yields of *A. niger* strain M in the absence of Mn, and Sarawathi-Devi (1954, 1962) obtained 120 % growth in Mn-deficient cultures as compared to the 'plus all' cultures. The higher mycelial yields in the absence of Mn might probably be explained by the fact that the mycelial dry weights were measured on the 6th day when 'plus all' cultures had sporulated abundantly and hence gave a lower mycelial dry weight than the Mn-deficient cultures which showed neither sporulation nor decrease in mycelial yields. Donald *et al.* (1952*b*) indicated the inefficiency of the purification procedure adopted by Nicholas (1950, 1952). Presumably this was only one of the contributory factors for masking of the Mn-deficiency symptoms, since Sarawathi-Devi (1954, 1962) had used the more efficient purification method of Donald *et al.* (1962*b*) which was also used in the present work. A feature common to all the investigations in which the absence of Mn gave no decrease in mycelial yields was the use of a high salt concentration in the basal medium (about 8.75–9.5 g./l.) and the addition of very high concentrations of micronutrient elements. A high salt concentration, although resulting in tough twisted mycelial strands instead of smooth fluffy growth, does not prevent the decrease in mycelial yields in the absence of Mn, although Donald *et al.* (1952*b*) did obtain more than 60 % decrease with a concentration of 8 g./l. However, it is evident from the data of Steinberg (1936) that increasing the concentration of trace elements beyond an optimum value either at pH 7.35 or at pH 8.04 resulted in the lowering of the growth ratio (yield with element : yield without element) besides having a toxic effect on sporulation. Hence it is probable that a high trace element concentration also contributes to masking the Mn-deficiency symptoms.

The comparison of the response of the five strains of *Aspergillus niger* to Mn concentration indicates that although all the strains showed a graded increase in mycelial yields, the effective range for assay of Mn was greater with strain MUBL I than with the other strains tested, the range lying between 0.001 and 0.006 $\mu\text{g. Mn/ml.}$ If sporulation could be measured precisely, the range of concentrations between 0.006 and 0.02 $\mu\text{g. Mn/ml.}$ could also be included for purposes of assay.

Aspergillus niger MUBL I strain appears to be an ideal test organism for bioassay of manganese. This method has been found to be 100-fold more sensitive than the well known spectrophotometric method with potassium periodate or ammonium persulphate as the oxidizing agent for conversion of Mn into MnO_4 (Sulochana, 1967).

Under the same cultural conditions the possible use of strain MUBL I for Cu and Mo assays was also studied; satisfactory results were obtained even for Mo series for which neither Al_2O_3 purification nor the H_2S co-precipitation method of Hewitt & Hallas (1951) was reported to be satisfactory (Saraswathi-Devi, 1954).

Aspergillus niger MUBL I has been used for assay of Mn in soils, plant tissues, human tissues, sera and blood; the standard series repeated every time with the samples have been consistently reproducible. The results of bioassay of manganese in cotton-growing soils will be reported elsewhere.

Grateful thanks are due to Professor T. S. Sadasivan for his interest and encouragement, and to Professor T. C. Vanterpool for critically reading the manuscript. We are thankful to the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A., for lyophilized cultures of *Aspergillus niger* strains NRRL 323, 334 and 346. Thanks are due to Indian Council of Medical Research and the Indian Council of Agricultural Research for grants during the tenure of which this work was done.

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