NUTRITIONAL STUDIES ON PIRICULARIA ORYZAE[1]

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Piricularia oryzae Cav., the cause of the disease known as "rice blast" which is commonly found in many of the rice-growing areas of the world, varies considerably in quantity and quality of growth on natural media, as shown by Henry and Andersen (1944). This paper is a report of the development of chemically defined ("synthetic") media for *P. oryzae* with the aim of producing growth and sporulation comparable to the best natural media and of reducing the degree of variation in conidia production on subculture below that found when the fungus is grown on a natural medium such as rice polish agar.

Little is known of the nutritional requirements of *P. oryzae*. Tochinai and Nakano (1940) reported growth on a synthetic medium containing only NH_4NO_3, $MgSO_4$, xanthine, glucose, and inorganic salts. Attempts in this laboratory to confirm their work were unsuccessful.

METHODS

A simplified medium (chemically defined except for the presence of purified agar and acid-hydrolyzed "vitamin-free" casein) was prepared for the cultivation of the fungus. All ingredients were included which are commonly required by fungi, and several compounds were added which had shown evidence of being beneficial in preliminary experiments (table 1). The usual precautions as to cleanliness of glassware and purity of reagents necessary in nutritional studies were observed.

The vitamin-free casein was hydrolyzed with H_2SO_4, which was subsequently nearly neutralized with $Ba(OH)_2$ to remove all but a small amount of sulfate. After the precipitate was washed with distilled water, the hydrolyzed casein solution was clarified with charcoal at pH 3.5 to 4.0 until it was nearly colorless. The concentrations of hydrolyzed casein reported in the tables were computed on the basis of the amino nitrogen content of the hydrolyzates. A bacteriological assay[4] of the medium (table 1) with *Lactobacillus casei* showed that it contained no biotin, no pantothenic acid, and approximately 0.15 μg per ml of nicotinic acid. A chemical assay[4] showed less than 0.04 μg per ml of thiamine.

Commercial bacteriological agar was washed three times with a mixture of equal parts of pyridine and ethyl alcohol, then with distilled water until no trace

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[4] Analysis by Capt. D. H. Bornor, AUS.

of pyridine could be detected by odor, and finally washed three more times with distilled water. It was dried at 50 C for a minimum of 3 days. All of the growth media reported in this paper were solidified with 2.0 per cent of this purified agar.

All cultures were grown in selected 18-by-150-mm pyrex culture tubes in which the media were slanted uniformly. The average area was found to be 9.6 cm^2, with variations of not more than 0.5 cm^2 among individual tubes.

The stock cultures of *P. oryzae*[5] were grown on 2 per cent rice polish, 2 per cent agar slants. Conidia from several 5- to 6-day-old cultures were suspended in sterile distilled water by violent shaking. The suspensions were combined and used for inoculating the media in each experiment. This method produced a low concentration of conidia, but the suspension was relatively free from mycelial fragments and undesirable material from the rice polish agar slant. Ten to 20 thousand conidia suspended in 0.5 ml of sterile distilled water were added to each slant by means of a syringe. The suspension was distributed over the entire surface of the slant by tilting and the excess liquid absorbed on the cotton plug. Repeated tests showed that for the size of the cotton plug used, the amount of excess liquid absorbed was so small that no contamination occurred because of the wetting of the plug. Uniform growth occurred over the agar surface, and the variation between replicate tubes was held to a minimum.

Four replicates of each medium were inoculated and incubated in the dark for 5 days. In the early experiments, incubation was at 22 to 28 C; in later experiments incubation was at 25 to 27 C. The amount of growth obtained was estimated visually. The degree of sporulation was determined microscopically by counting in a Howard chamber the sample obtained by suspending the conidia in each tube in 10 ml water. The conidia counts given in this paper are averages of four replicate tubes. All results on vitamin requirements were analyzed statistically.

RESULTS

Vitamin requirements. The requirements for the B vitamins were determined by adding the following vitamins of the B complex to the basal medium (table 1): thiamine hydrochloride, calcium pantothenate, nicotinic acid, biotin, *d*-riboflavin, and pyridoxine. To test the effects of these vitamins, they were omitted singly and in groups from a medium containing all of these six vitamins. The omission of nicotinic acid, riboflavin, pyridoxine, and calcium pantothenate had no significant effect on the yield of conidia; however, when either thiamine or biotin was omitted no growth occurred. The results are summarized in table 1. Other experiments showed that neither *p*-aminobenzoic acid nor a folic acid concentrate[6] affected the growth or production of conidia of *P. oryzae*.

Six levels of biotin were added to a medium containing only chemically known ingredients other than agar to determine the optimal biotin level for growth and

[5] The culture of *P. oryzae* was obtained from Dr. E. C. Tullis, U. S. D. A., Beaumont, Texas.

[6] We wish to thank Dr. R. J. Williams, University of Texas, for the gift of this folic acid concentrate.

production of conidia. The results (table 2) indicate that the optimal level lies between 0.001 and 0.01 μg per ml. This experiment confirmed, in a more highly purified medium, the previous findings that no B complex vitamins other than thiamine and biotin are required.

An attempt was made to replace biotin with cysteine and pimelic acid, as Eakin and Eakin (1942) have done with *Aspergillus niger*. *P. oryzae* did not grow on the basal medium when either cysteine (50 μg per L) or pimelic acid (65 μg per L) or a combination of both was added in place of biotin.

TABLE 1

Effect of B complex vitamins on growth and production of conidia by P. oryzae

VITAMIN OMITTED FROM MIXTURE*	GROWTH	SPORES IN THOUSANDS/CM²		
		Subculture no.		
		1	2	3
None	good	16.7		
Nicotinic acid	good	10.3		
Ca-pantothenate	good	11.1		
d-Riboflavin	good	14.0		
Thiamine·HCl	none	0.0		
Pyridoxine	good	12.2		
Biotin	none	0.0		
None	good	108	430	81
Nicotinic acid, Ca-pantothenate, *d*-Riboflavin, Pyridoxine	good	102	670	203
Rice polish agar control	good	62	81	59

Medium (g/L): sucrose, 5.0; acid-hydrolyzed "vitamin-free" casein, 1.0; agar, 20; K_2HPO_4, 0.5; glycerol, 0.05; oleic acid, 0.05; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCO_3$, 0.05; Na_2CO_3, 0.05; *i*-inositol, 0.04; guanine, 0.05; xanthine, 0.05; uracil,† 0.1; guanidine·HCl, 0.05; $CuCl_2$, 0.0001; 85% H_2MoO_4, 0.00001; H_3BO_3, 0.0005; $MnSO_4$, 0.001; $ZnCl_2$, 0.0005; $Fe(NH_4)_2(SO_4)_2$, 0.0005. pH 6.5 ± 0.1.

* Vitamin mixture (μg/ml): nicotinic acid, 7.0; Ca-pantothenate, 2.5; *d*-riboflavin, 2.5; thiamine·HCl, 2.0; pyridoxine, 1.0; biotin, 0.01.

† Synthesized by T/5 W. L. Mosby.

In order to determine whether *P. oryzae* can be continuously cultivated in simplified media, the fungus was carried for 6 successive subcultures on a chemically defined agar medium and the growth and conidia production compared with 6 corresponding transfers on a 2 per cent rice polish, 2 per cent agar medium. The degree of variation in sporulation on subculture in the two types of media was analyzed statistically. The results are presented in table 3. It is apparent that a good chemically defined medium supports adequate sporulation with less variation on subculture than does the natural medium.

Nitrogen requirements. The requirements of *P. oryzae* for amino nitrogen were studied by the omission of each amino acid from a medium containing a mixture of 15 amino acids. The mixture contained glycine, *l*(+)lysine, *dl*-valine,

l(−)leucine, *dl*-isoleucine, *dl*-threonine, *dl*-phenylalanine, *dl*-methionine, *dl*-glutamic acid, *dl*-aspartic acid, *l*(−)proline, *l*(−)hydroxyproline, *l*(+)arginine, *l*(−)tryptophane, and *l*(+)histidine in concentrations equivalent to their pro-

TABLE 2

Determination of optimal biotin level for growth and conidia production by P. oryzae

BIOTIN (μG/ML)	SPORE COUNT IN THOUSANDS PER CM²	VISUAL ESTIMATION OF GROWTH
0.0	0	none
0.00001	0	very slight
0.0001	4.2	poor
0.001	1,220	good
0.01	1,880	good
0.02	1,610	good

Basal medium (g/L): glucose, 5.0; acid-hydrolyzed "vitamin-free" casein, 1.0; agar, 20; 62% potassium glycerol phosphate, 0.9; $MgSO_4 \cdot 7H_2O$, 0.5; *i*-inositol, 0.02; guanine, 0.0066; xanthine, 0.0066; uracil, 0.0066; guanidine·HCl, 0.0066; choline·Cl, 0.001; thiamine·HCl, 0.002; $CuCl_2$, 0.0001; 85% H_2MoO_4, 0.0001; H_3BO_3, 0.0005; $MnSO_4$, 0.001; $ZnCl_2$, 0.0005; $Fe(NH_4)_2(SO_4)_2$, 0.0005. pH 6.5 ± 0.1.

TABLE 3

Comparison of variations of conidia production upon subculture of P. oryzae on a rice polish medium and a chemically defined medium

SUBCULTURE NO.	2% RICE POLISH, 2% AGAR MEDIUM CONIDIA IN THOUSANDS/CM²	CHEMICALLY DEFINED MEDIUM* CONIDIA IN THOUSANDS/CM²
1	1,700	896
2	771	1,250
3	1,860	615
4	2,720	844
5	760	760
6	292	781
Total	8,100	5,150
Average	1,350	858
Mean deviation between subcultures	66.3%	24.9%

* Medium (g/L): glucose, 5.0; agar, 20; $K_4P_2O_7$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.05; Na_2CO_3, 0.05; glycerol, 0.05; thiamine·HCl, 0.002; choline·Cl, 0.001; biotin, 0.00001; *i*-inositol, 0.02; guanine, 0.0066; xanthine, 0.0066; uracil, 0.0066; guanidine·HCl, 0.0066; *l*(−)tryptophane, 0.0193 (0.0001 M); *dl*-glutamic acid, 0.0144 (0.0001 M); *l*(−)leucine, 0.013 (0.0001 M); *l*(−)proline, 0.0117 (0.0001 M); *l*(+)histidine, 0.0154 (0.0001 M); glycine, 0.788 (to raise amino nitrogen to level equivalent to 0.1% casein); $CuCl_2$, 0.0001; 85% H_2MoO_4, 0.00001; H_3BO_3, 0.0005; $MnSO_4$, 0.001; $ZnCl_2$, 0.0005; $Fe(NH_4)_2(SO_4)_2$, 0.0005. pH 6.5 ± 0.1.

portions in 0.1 per cent casein. The single omission of each of the amino acids from the medium made little difference in growth or conidia production. In later work it became apparent that any one of several amino acids could function equally well as a nitrogen source, provided a concentration at least equal to the

amino nitrogen of 0.1 per cent casein was used. The results of this work, including 6 successive subcultures, are presented in table 4. It is evident that *P. oryzae* can be maintained in subculture in media such as those given in table 4.

TABLE 4

Effects of various amino acids and of $(NH_4)_2SO_4$ as nitrogen sources in the continuous cultivation of P. oryzae

NITROGEN SOURCE	g/L	SPORES IN THOUSANDS/CM²					
		Subculture no.					
		1	2	3	4	5	6
Casein	1.0	310	247	55.3	101	223	111
$(NH_4)_2SO_4$*	0.75	4.2	7.0	2.1	2.1	3.5	5.6
Glycine†	0.86	213	334	56.0	98.7	89.6	244
dl-Tryptophane	0.204	83.3	134	179	56	104	200
Glycine†	0.7						
dl-Glutamic acid	0.147	101	103	128	125	119	120
Glycine†	0.7						
dl-Leucine	0.131	82.6	94.5	129	62.3	76.3	132
Glycine†	0.7						
dl-Tryptophane	0.204						
dl-Glutamic acid	0.147	43.4	82.6	26.6	22.8	35.0	72.8
dl-Leucine	0.131						
Glycine†	0.42						
l(−)Tryptophane	0.0204						
dl-Glutamic acid	0.0147						
l(−)Leucine	0.0131	120	179	302	82.6	229	252
l(−)Proline	0.0115						
l(+)Histidine	0.0155						
Glycine†	0.79						

Medium (g/L): glucose, 5.0; agar, 20; 62% potassium glycerol phosphate, 0.9; $MgSO_4 \cdot 7H_2O$, 0.001; $CaCl_2$, 0.0005; Na_2CO_3, 0.0005; *i*-inositol, 0.02; guanine, 0.0066; xanthine, 0.0066; uracil, 0.0066; guanidine·HCl, 0.0066; choline·Cl, 0.001; thiamine·HCl, 0.002; biotin, 0.00001; $CuCl_2$, 0.0001; 85% H_2MoO_4, 0.00001; H_3BO_3, 0.0005; $MnSO_4$, 0.01; $ZnCl_2$, 0.0005; $Fe(NH_4)_2(SO_4)_2$, 0.0005. pH 6.5 ± 0.1.

* Conidia produced in $(NH_4)_2SO_4$ media were morphologically abnormal and failed to germinate. Subcultures in $(NH_4)_2SO_4$ media were made by mycelial transfer.

† Glycine was added in each experiment to raise the concentration of amino nitrogen to that of 0.1% casein.

Experiments were then undertaken to determine the various sources of organic nitrogen, other than amino acids, which are available to this fungus. Two basal media were used, one containing no organic nitrogen (except that in 0.01 μg per ml biotin and 2.0 μg per ml thiamine·HCl) and the other containing 12.5 μg per

ml of total organic nitrogen of which 3.7 μg per ml were amino nitrogen. The compounds to be tested were all added to the basal medium in 0.1 per cent concentration. The results are presented in table 5. Although a large number of widely diverse nitrogenous compounds supported growth and conidia formation,

TABLE 5

Effects of various nitrogen sources on the growth and sporulation of P. oryzae

TEST COMPOUND*	"NITROGEN-FREE" BASAL MEDIUM†		"NITROGEN-FREE" BASAL MEDIUM PLUS MIXTURE OF NITROGENOUS NUTRIENTS‡		
	Growth	Conidia in thousands/cm²	Growth	Conidia in thousands/cm²	
				1	2
Casein	+++++	790	++++	235	20.0
Glycine	++++	113	+++	409	369
Betaine	+	0	+++	83.3	61.6
ε-NH_2-caproic acid	+	0	++	31.5	74.2
Guanidine·HCl	+	0	+++	83.3	57.4
Urea	++	7.0	+++	110	72.8
Thiourea	0	0	0	0	0
Nicotinic acid	+	0	++	41.3	46.2
p-NH_2-benzoic acid	+	0	++	0	3.5
Uracil	++	7.0	+++	10.5	55.3
Uric acid	+++	14.0	+++	67.9	120
Caffeine (citrated)	+	0	++	78.4	32.2
Triethanolamine	+	0	+++	34.3	63.0
Ethanolamine	+	0	+++	20.3	35.0
Choline chloride	+	0	+++	44.8	36.4
Hydroxylamine	0	0	0	0	0
NH_4Cl	0	0	+		9.8
None	0	0	+		37.1

* All compounds except NH_4Cl were tested at 0.1% concentration. The pH was adjusted to 6.5 ± 0.1 before inoculation of the media. NH_4Cl was tested at 0.06% concentration, equivalent to 0.1% casein.

† "Nitrogen-free" basal medium (g/L): glucose, 5.0; 62% potassium glycerophosphate, 0.9; agar, 20; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCO_3$, 0.05; Na_2CO_3, 0.05; *i*-inositol, 0.02; thiamine·HCl, 0.002; biotin, 0.00001; $CuCl_2$, 0.0001; 85% H_2MoO_4, 0.00001; H_3BO_3, 0.0005; $MnSO_4$, 0.001; $ZnCl_2$, 0.0005; $Fe(NH_4)_2(SO_4)_2$, 0.0005. pH 6.5 ± 0.1.

‡ Mixtures of nitrogenous nutrients (μg/ml in final medium): guanine, 6.6; xanthine, 6.6; uracil, 6.6; guanidine·HCl, 6.6; choline·Cl, 1.0.

α-amino acids were required for full activity. The amino acid requirement was apparently satisfied by glycine alone.

With the medium (table 4) containing the six amino acids (glycine, tryptophane, histidine, leucine, proline, and glutamic acid), choline, inositol, guanine, guanidine, uracil, and xanthine were omitted from the medium singly and as a group to determine whether any of these compounds are essential in an amino acid basal medium. The results (table 6) indicate that none of these compounds are essential for growth or conidia production during a period of 4 subcultures.

Some of the compounds named may be beneficial in stabilizing the growth and conidia production of the organism.

Some of the media were altered for use in submerged cultures by reducing the content of agar to 0.1 per cent. Heavy growth was observed, but conidia were not formed in liquid cultures incubated in aerated bottles. No attempts were made to induce conidia formation in submerged cultures by nutritional alterations. A shaking machine for aeration by agitation was not available during this work; its use might prove valuable in producing submerged sporulated cultures.

TABLE 6

Effects of certain accessory growth factors on growth and production of conidia by P. oryzae

COMPOUND OMITTED FROM MEDIUM	SPORES IN THOUSANDS/CM²			
	Subculture no.			
	1	2	3	4
None	222	1,420	362	998
Choline·Cl	78.4	778	1,350	511
Inositol	81.2	491	314	330
Guanidine·HCl	109	312	167	360
Guanine	95.2	225	1,450	750
Xanthine	104	557	1,130	213
Uracil	137	878	918	692
Choline·Cl, Inositol, Guanine, Xanthine, Uracil, Guanidine·HCl	146	347	1,280	943

Medium: Same as given in table 4 including the six amino acids listed together. The compounds named above were used in the concentrations given in table 4.

Conidia of *P. oryzae* produced in chemically defined media have shown 97 to 99 per cent germination[7] and have been found as infective[8] for the rice plant in the greenhouse as conidia produced on natural media.

DISCUSSION

On the basis of these studies, the nutritional requirements of *P. oryzae* appear to be relatively nonspecific except with regard to the vitamins, thiamine and biotin being the only ones required. This fungus requires organically combined nitrogen (preferably α-amino acids), but a large number of compounds in which the nitrogen exists in amino, cyclic, imino, or quaternary combination will support growth and conidia formation. No complete investigations were made of the essentiality of some of the other components of the medium, especially the inorganic ions.

[7] We wish to thank Capt. J. W. Marek, AUS, for performing the germination experiments.

[8] We wish to thank S/Sgt. T. L. Morgan, AUS, for performing the infectivity experiments.

Although the fungus grew more uniformly on the chemically defined media than on natural media, such as rice polish agar, the degree of variation was considerable with all media. The variability of the quantity of growth in replicates prevents accurate evaluation of nutrients the effects of which are quantitatively of a low order. Perhaps further nutritional investigations would lead to greater uniformity, especially if submerged dispersed growth in liquid media could be used in place of surface growth. Although the production of conidia was less variable on an adequate chemically defined medium than on a natural medium (table 3), when certain of the pure nutrient compounds were omitted from chemically defined media to determine their essentiality (tables 4 and 6) the resulting cultures were sometimes as variable as those on rice polish agar.

SUMMARY

Thiamine (2 μg per ml or less) and biotin (0.01 μg per ml) are required for growth and conidia formation by *Piricularia oryzae*. Other B complex vitamins are not required.

P. oryzae requires organically combined nitrogen, preferably α-amino acids, but can use many types of organic compounds in which the nitrogen exists in amino, imino, cyclic, or quaternary combination.

P. oryzae can be maintained successfully in subculture on chemically defined media, the degree of variation and the yields, viability, and the degree of germination and infectivity of conidia comparing favorably with cultures grown on natural media.

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