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Studies on *Diplodia* and *Diplodia*-like Fungi

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Fifteen isolates representing various genera and species of *Diplodia* and *Diplodia*-like fungi were grown on various synthetic media, for study of the effect of pH, temperature, light, and vitamins on growth, sporulation, and stability of morphological characters currently used to delimit members of the Phaeodidymous Sphaeropsidales taxon. Fungi tested grew over a wide pH range. A bimodal response in growth at pH levels near 4.5 and 7.0 was common for most but not all isolates tested. The pH of the culture medium within ranges allowing good growth had little influence on mycelial color or general colony appearance. Sporulation was influenced by pH, however, apparently more so by the buffering system. Although the pH of the culture medium influenced production of fruiting structures and spores, it had little effect on stabilizing characteristics used in classification of these fungi.

Temperatures ranging from 6° to 39°C had the usual expected effects. Growth of isolates increased as temperature increased, peaked at a range from 27° to 33°C, and then dropped rapidly to form a skewed curve. Temperature apparently had little influ-

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IV. Effects of pH, Temperature, Light, and Vitamins on Certain Taxonomic Characters[1, 2]

ABSTRACT

Fifteen isolates of *Diplodia* and *Diplodia*-like fungi were grown on various synthetic media for study of the effects of pH, temperature, light, and vitamins on growth, sporulation, and stability of morphological characters currently used in identification. The pH of the culture medium within ranges for good growth had little effect on stabilizing the characteristics used in classification. Light, either continuous or cyclic, favored growth and maturity more than did continuous dark, although some isolates did grow under the latter condition. Two isolates required biotin for sporulation. All others tested grew and sporulated on minimal medium without biotin or other added vitamins. Potato-dextrose-agar is considered a satisfactory medium for growing these fungi for identification purposes.

INTRODUCTION

EARLIER STUDIES show that taxonomic criteria currently used to delimit *Diplodia* and *Diplodia*-like fungi are often variable under experimental conditions (Satour, Webster, and Hewitt, 1969*a, b;* Webster, Hewitt, and Polach, 1969).

The objective of our studies is to determine a standard culture medium or a set of conditions that might stabilize characteristics for use in the taxonomy of this group. Studies on effects of environment and nutrition are therefore viewed in this light.

This paper reports on the effects of pH of the culture media, light, temperature, and certain vitamins on taxonomic characters used at present in separation of this group. Criteria evaluated include: range of probable alterations induced by the variables on growth, cultural characteristics, color of colonies, gross changes in morphology of hyphae and fruiting bodies, and on the production of fruiting bodies and spores. No attempt is made to evaluate such physiological aspects as may be evident in the results observed.

MATERIALS AND METHODS

Isolates of genera and species used in this study (table 1) are part of a collection of some 250 isolates of fungi that belong to the Phaeodidymous: Sphaeropsidales taxon, which may be found in the Department of Plant Pathology at the University of California, Davis.

[1] Submitted for publication October 26, 1970.

[2] The investigations covered by all papers were supported by Public Health Service Research Grant No. UI-00298.

TABLE 1

HOST, LOCATION, AND SOURCE OF FUNGUS ISOLATES

Isolate no.	Taxon	Host	Location	Source
6	*Diplodia natalensis*	Citrus fruit	.....	A.T.C.C. #9055*
19	*Diplodia* sp.	Persimmon fruit	California	U.C.C.C. #1214†
29	*Botryodiplodia hypodermia*	.....	.....	C.B.S. #6318‡
35	*Diplodia macrospora*	.....	.....	C.B.S.
44	*Botryodiplodia theobromae*	Banana fruit	.....	A.T.C.C. #16391
55	*Botryosphaeria ribis*	.....	.....	C.B.S.
86	*Physalospora rhodina*	Citrus fruit	.....	A.T.C.C. #10936
107	*Diplodia natalensis*	Citrus fruit	Brazil	M.M.§
130	*D. zeae*	Corn	Illinois	A. L. Hooker #6
147	*D. natalensis*	Citrus fruit	Egypt	K. Y. Mickail #1
157	*D. natalensis*	Grape	California	Present authors
200	*D. natalensis*	Grape	California	Present authors
213	*D. natalensis*	Grape	California	Present authors
218	*Sphaeropsis* sp.	Grape	California	Present authors
230	*Diplodia natalensis*	Grape	California	Present authors

* American Type Culture Collection, Washington, D.C.
† University of California Culture Collection, Davis, California.
‡ Central Bureau for Fungus Culture, Baarn, Netherlands.
§ Mycological Museum, Paris, France.

Culture media[3]

POTATO-DEXTROSE-AGAR (PDA)

COMPOSITION

200 gm potatoes
1 liter water
10 gm glucose
15 gm agar-agar

Potatoes were peeled, diced to ½-inch cubes, added to water, and cooked for 30 minutes at just below boiling point. The liquid was strained through cheesecloth. Glucose and agar were added, and the liquid was autoclaved for 15 minutes at 121°C and then adjusted to the desired pH level with NaOH and HCl.

SYNTHETIC MEDIUM A COMPOSITION

	gm/l
Glucose	10.0
Potassium nitrate	2.0
Potassium chloride	0.5
Magnesium sulfate	0.5
Ferrous sulfate	0.001
Yeast extract	2.0
Double-distilled water to make 1 liter	

Solid media were prepared by adding 15 gm of Bacto-agar to each liter of culture media. The mixture was autoclaved for 15 minutes at 121°C.

After autoclaving, the pH was adjusted to desired levels with NaOH and HCl or, for some experiments, with citrate, phosphate, carbonate, acetate, and Tris buffer systems:

SYNTHETIC MEDIUM B COMPOSITION

Solution 1:

Water (deionized) 1 liter
Glucose, 1 gm
L glutamic acid, 1 gm
D-L methionine, 400 mg
Thiamine, 20 mg

Solution 2:	*mg/l*
Ferric chloride	156
Manganese chloride	114
Zinc sulfate	27
Molybdenum oxide	34
Calcium nitrate	98
Copper sulfate	250
Calcium chloride	220
Boric acid	280
EDTA	8
Magnesium sulfate: $7H_2O$	200
Water (deionized) to make 1 liter	

The culture medium was prepared by adding 10 ml of solution 2 to each liter of solution 1.

[3] Culture medium for the vitamin studies is given on p. 90.

The desired pH level was attained by buffering with dibasic and monobasic phosphate solutions. Media were sterilized by passing through a Millipore filter.

Inoculating plates

Unless otherwise stated, 15 ml of culture medium were added to each petri dish. Cultures were inoculated at the center of the plate with a 3-ml plug of mycelium from the margin of a 4- to 5-day-old culture on water agar.

Numbers of pycnidiospores produced were determined by homogenizing the entire contents of a petri dish culture in 80 ml of water, with a Waring Blendor, for about one-half minute, raising the final volume to 100 ml, and calculating the numbers of spores from counts of samples by means of a hemocytometer.

TABLE 2

AVERAGE COLONY DIAMETERS* OF ISOLATES OF *DIPLODIA* AND RELATED GENERA ON POTATO DEXTROSE AGAR INITIALLY ADJUSTED TO DIFFERENT pH VALUES

(Cultures were grown for 4 days at 24°C, under fluorescent light, daylight type, 60 ft-c, 8 to 9 hours daily.)

pH	Colony diameters (*mm*)									
	Isolate no.									
	6	44	55	86	107	130	157	213	218	230
3.0	20	42	19	29	19	33	55	59	13	34
4.0	34	89	26	79	37	35	89	84	65	81
4.5	36	89	27	78	42	46	89	90	54	75
5.0	40	87	30	84	45	44	90	90	64	90
5.4	36	81	36	71	43	40	73	79	52	89
6.2	36	76	41	64	42	38	71	80	53	85
6.6	36	68	38	68	39	32	71	80	49	74
7.1	39	88	36	67	42	27	83	80	54	82
8.1	36	67	36	66	34	18	72	70	44	64
9.0	35	67	30	51	35	9	62	61	39	55
10.0	24	49	24	44	25	7	49	50	26	31
11.0	19	37	21	21	16	7	40	40	21	27

* Average of three plates.

EFFECTS OF pH

Growth at different pH levels on different media

Table 2 shows the growth of 10 isolates on PDA at initial pH levels ranging from 3 through 11. Growth was determined as a mean of the colony diameter on three petri dishes on the fourth day after inoculation. The pH of the growth media in the petri dishes was not determined on the fourth day because the cultures were to be held for 30 days to allow fruiting structures to mature.

Two growth maximums were observed for each of eight of the 10 fungus isolates, one at pH level near 4.5 to 5, the other at 7.1. The maximum growth of isolate number 55 occurred at pH 6.2. Isolate 213 grew well over a broad range of pH values from 4 through 7.

Table 3 shows the mean diameter of colonies after five days' growth on medium A buffered separately with citrate, phosphate, and carbonate systems, over a range of pH 2.5 through 10, and on the same medium adjusted

TABLE 3

AVERAGE COLONY DIAMETERS* OF ISOLATES OF *DIPLODIA* AND RELATED GENERA GROWN ON SYNTHETIC MEDIUM INITIALLY ADJUSTED TO DIFFERENT pH VALUES

(Cultures were grown for 5 days at 24°C under fluorescent light, 60 ft-c, 8 to 9 hours daily.)

Buffer and initial pH	Colony diameters (*mm*)									
	Isolate no.									
	19	29	35	44	86	130	147	157	213	230
Citrate:										
2.5	48	0	0	52	40	0	47	51	36	46
3.2	90	27	49	90	90	82	90	90	90	90
4.1	90	32	54	90	90	90	90	90	90	90
Phosphate:										
5.7	83	19	12	73	39	7	90	58	71	77
7.0	33	0	7	31	29	0	39	34	31	33
7.7	16	0	0	18	15	0	19	20	18	18
Carbonate:										
8.9	77	0	0	68	76	0	82	84	74	64
9.2	69	0	0	49	63	0	76	77	68	60
10.0	68	0	0	48	62	0	62	66	50	59
Acetate:										
3.6	0	0	0	0	0	0	0	0	0	0
4.6	0	0	0	0	0	0	0	0	0	0
5.6	66	0	0	68	65	0	74	0	59	68
Tris:										
7.2	90	24	7	88	89	9	90	90	90	76
8.2	88	35	7	84	90	8	90	89	90	76
9.0	68	31	6	82	88	2	90	81	85	73

* Average of three plates.

to pH levels of 3.6 through 9 with acetate and Tris buffers. Bacto-agar was added to solidify the media. Five of the same isolates shown in table 2 (44, 86, 157, 213, and 230) and two additional isolates, 19 and 147, showed highest growth rates at two pH levels, 3.2 and 4.1. Isolates 29, 35, and 130 grew poorly on the citrate, phosphate, and carbonate buffer systems, but did grow on Tris-buffered media at high pH values. None of the isolates grew on media buffered with acetate at pH 3.2 and 4.6, whereas they all grew on plates buffered with Tris.

The rate of growth of the isolates over the fourth and fifth days as measured by colony diameter on solid-culture media varied with the isolate, the type of medium, the initial pH at the time of inoculation, and the buffer system.

In another experiment with liquid medium A buffered with citrate at pH 2.5 to 4.1, phosphate at pH 5.7 to 7.7, and carbonate at pH 8.9 to 9.2, isolate 157 had only one maximum growth rate at pH 7 to 7.7. In this experiment there was more growth per day, expressed as dry weight of mycelium, with tests terminated on the eighth day than with tests stopped on the fourth or twelfth days. Isolate 55 produced the greatest growth per day at pH 5.7.

Growth measured by colony diameter on solid media may or may not appropriately represent the over-all growth of a fungus mycelium in culture. Furthermore, the bimodal growth response obtained at different initial pH levels on solid media was not obtained by isolates 157 and 55 in liquid culture buffered at pH levels from 2.5 to 9.2. Therefore, isolates 157 and 200 of *D. natalensis* were grown in liquid medium B adjusted to pH levels 2.5 through 9.5 with mono- and dibasic phosphate. The apparatus used permit-

ted changing the culture solution daily. Culture flasks were inoculated with 1 ml of spore suspension containing approximately 40,000 spores. The entire culture medium in each flask was changed daily and renewed with new culture solution from the original lot at the initial pH level.

Figure 1 shows the mean growth (μg dry weight) of mycelium per day produced over an 18-day period from the time of seeding. Each experiment was replicated three times at each pH level. In these tests, isolate 200 had maximum growth rates at pH levels 4 and 8. Table 4 shows the pH of the initial culture solution and the pH of the culture solution withdrawn from the mycelium mat at each daily interval over the 18 days of growth, for one experiment. Data for other experiments were similar. There was little daily change in pH at the lower levels of 2.5, 3, and 4. At higher pH levels, 5 through 9.5, the pH of the culture solution shifted during each 24-hour period to slightly lower levels than that of the initial pH.

In a similar experiment, growth of isolate 157 followed very closely that shown for isolate 200.

It is interesting to note that isolate 200 produced a soluble red pigment only in media initially adjusted to pH 8 and that ranged from 7.6 to 7.8 on the sixth through the seventeenth day of the experiment.

The daily shifts in the pH of the culture solution shown in table 4 indicate that the fungus very likely shifted

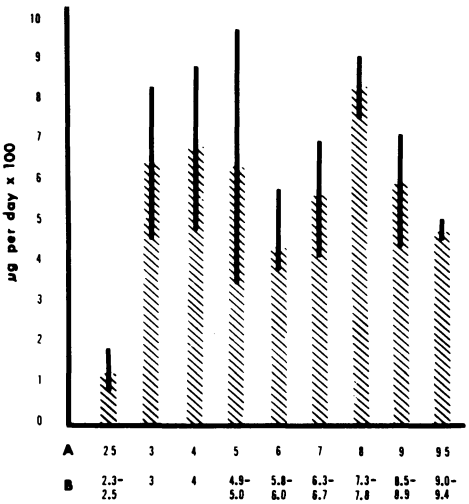

Fig. 1. Mean growth (μg dry weight) per day of mycelium of *Diplodia natalensis* 200 in liquid media adjusted to different pH levels. Culture solutions were replaced daily with initial solution. (See table 4 for pH values of culture solutions at time of removal over 18-day period.) Bars are means of three experiments; solid lines are extremes. Each experiment was replicated three times. A = initial pH each day; B = range of pH of culture solution withdrawn at end of each day.

TABLE 4

pH OF CULTURE SOLUTIONS OF *DIPLODIA NATALENSIS* 200 WITHDRAWN AT DAILY INTERVALS

(Withdrawn solution was replaced with initial culture solution at given pH value, 0 time. See Fig. 1 for weights.)

Initial pH	pH at following days after seeding:																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2.5	2.5	2.3	2.4	2.4	2.4	2.4	2.4	2.5	2.4	2.4	2.4	2.4	2.4	2.4	2.4	...	...	...
3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	...	...	...
4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	...	...	...
5.0	4.9	4.9	4.9	4.9	4.9	5.0	5.0	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	...	...	...
6.0	6.0	5.9	5.9	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.7	...	...	...
7.0	...	...	6.6	6.4	6.4	6.6	6.5	6.6	6.3	6.3	6.5	6.5	6.3	6.3	6.5	6.6	6.6	...
8.0	...	...	7.4	7.3	7.6	7.7*	7.8	7.7	7.6	7.6	7.7	7.7	7.7	7.7	7.7	7.8	7.8	...
9.0	...	...	8.6	8.5	8.8	8.7	8.8	8.8	8.8	8.8	8.8	8.8	8.7	8.7	8.7	8.9	8.9	...
9.5	...	...	9.0	8.8	9.3	9.1	9.2	9.2	9.2	9.2	9.2	9.2	9.1	9.1	9.2	9.3	9.4	...

* Isolate 200 produced a soluble red pigment after the fifth day through the seventeenth day only at initial pH 8, which ranged from 7.6 to 7.8 during that time.

TABLE 5

pH OF SAMPLES OF CULTURE SOLUTION UNDER A MAT OF MYCELIUM OF *DIPLODIA NATALENSIS* 200 AT DAILY INTERVALS FROM DATE OF SEEDING WITH CONIDIA

Initial pH	pH at following days after seeding:									Mycelium (dry wt.)
	1	2	3	4	5	6	7	8	9	
										μg
4	3.9	3.8	4.0	3.7	3.8	3.8	3.7	3.8	3.9	18.3
5	4.8	4.7	4.9	4.6	4.7	4.7	4.7	4.7	4.9	16.6
6	6.3	5.9	5.9	5.5	5.6	5.6	5.7	5.7	5.8	16.4
7	6.6	6.2	5.7	5.4	5.6	5.6	5.8	5.8	5.8	14.0
8	7.2	7.1	6.5	5.4	5.6	6.1	6.2	6.3	6.0	13.1

the pH of the solid culture media in earlier experiments. The probable daily change in pH of culture media was evaluated by growing isolate 200 in liquid synthetic medium B with l-tyrosine at the rate of 300 mg per liter. Cultures were grown in Erlenmeyer flasks seeded with a spore suspension of the fungus as before, except that the culture solutions were not changed during the nine days of the experiment. Cultures were grown under continuous fluorescent light in the laboratory at 20 to 23°C. Samples of 1 ml were withdrawn daily from a position just under the fungus mat, and the pH of each was determined. Table 5 shows the mean pH of three replications at each level for each of nine days and the total dry weight of mycelium mat at the end of the nine-day period.

In general, the pH of the culture media dropped slightly from the initial into the fourth day, then rose slightly, but remained lower than that of the initial through the ninth day. The higher the initial pH, the greater was the shift to the lower pH. For example,

TABLE 6

pH OF CULTURE MEDIA* AT INTERVALS DURING GROWTH OF *DIPLODIA NATALENSIS* 157 AND *BOTRYOSPHAERIA RIBIS* 55 IN LIQUID SYNTHETIC MEDIUM A

(Cultures were grown at 24°C under continuous fluorescent light at 250 ft-c.)

Species, isolate no., and buffer	pH at following days after seeding:						
	0	2	4	6	8	10	12
Botryosphaeria ribis 55:							
citrate	2.5	2.5	2.6	2.7	2.6	2.7	2.8
	3.2	3.3	3.4	3.7	3.9	4.1	4.3
	4.1	4.1	4.3	4.8	5.1	5.8	6.5
phosphate	5.7	5.7	5.7	6.0	6.1	6.4	6.5
	7.0	7.0	7.0	7.1	6.9	6.9	7.2
	7.7	7.8	7.8	7.9	7.9	7.7	7.7
carbonate	8.9	8.9	8.6	7.6	7.1	7.5	8.7
	9.3	9.2	9.2	9.2	9.0	8.6	8.5
Diplodia natalensis 157:							
citrate	2.5	2.5	2.6	2.6	2.6	2.7	2.7
	3.2	3.3	3.4	3.8	4.2	4.4	4.8
	4.1	4.1	4.5	4.9	5.4	5 7	5.9
phosphate	5.7	5.7	5.9	6.4	6.3	6.4	6.4
	7.0	7.0	7.0	7.0	6.7	7.0	7.1
	7.7	7.7	7.6	7.5	7.2	7.1	7.1
carbonate	8.9	8.8	7.4	6.5	8.1	8.4	8.7
	9.3	9.2	8.2	7.3	6.9	8.0	7.2

* Average of three replications.

the culture grown at pH 8 changed over four days to 5.4 and then shifted back to 6 to 6.3.

It is interesting to note that isolate 200 had a bimodal response to pH levels over this range in culture media that were changed daily (fig. 1; table 4). In the experiment in which the culture solution was not changed, however, the mycelium developed maximum weight only at pH 4 and not at the higher level.

In an experiment in which isolates *Botryosphaeria ribis* 55 and *D. natalensis* 157 were grown in liquid medium A buffered with citrate, phosphate, and carbonate, the pattern of change in pH of the culture solution was different from that described in the previous experiment. Table 6 shows the pH of the solutions at two-day intervals. At the lower initial levels (pH 2.5 to 5.7), the pH of the culture solution rose slightly over the 12-day period. At pH levels above 7, the pH of the culture solution dropped slightly over the same period and was, in this respect, similar to levels shown in table 5.

Studies of the growth of some isolates of *Diplodia* indicate that some of these fungi altered the pH of the solid medium at levels above 4 over the 30-day period of growth and sporulation. Changes from the initial pH above the level of 4 were generally to a lower level. At high pH levels near 8 and 9, the shift was often as much as 2 points. It is evident that the fungus can and indeed does shift the pH of the growth medium, both liquid and solid, and that the pH of the medium influences the growth of some of the isolates of *Diplodia* and *Diplodia*-like fungi.

Table 7 shows the status of stromata, pycnidia, and mode of spore dispersal of *D. natalensis* isolates 157 and 213, *B. theobromae* isolate 44, and *P. rhodina* isolate 86 grown on PDA at different initial pH levels, and buffered with NaOH and HCl. Table 8 shows similar data for the same four fungi grown on medium A buffered to different pH levels with citrate, phosphate, carbonate, acetate, and Tris. Data in tables 7 and 8 are typical of results obtained by growing 11 different isolates on the various media at different pH levels.

It is evident that characters used in the classification of these fungi varied with the different isolates, depending on the pH of the culture media (as initial pH at inoculation), and with the buffer used to adjust the pH of the culture media. These variations were evident in colony appearance, color, and manner of growth, production and distribution of pycnidia, location of pycnidia with respect to the medium, i.e., superficial or submerged, presence or absence of hairs on the pycnidia, size of separate pycnidia, and the manner of spore dispersal, i.e., whether wet or dry.

Table 9 shows the mean number of pycnidia per cm^3 and the mean number of spores per culture plate $\times 10^3$ for seven of the isolates grown on medium A buffered to different pH levels with citrate, phosphate, carbonate, acetate, and Tris. Isolate 29, not shown in the table, produced pycnidia and a few spores only at pH 6 in the phosphate-buffered medium, while isolate 130 sporulated only at pH levels 4 and 5 with citrate buffer.

Tables 3 and 9 indicate that the buffering systems also influenced growth and sporulation of some of these fungi as well as production of pycnidia and spores. Although the initial pH of the growth medium—PDA adjusted with NaOH and HCl—also had some influence on pycnidial formation in some of the isolates, for example, *B. theobromae* 44 (table 7), the degree was much less than that for isolates grown on media buffered with organic compounds.

In general, the range of variation was similar to that shown in earlier

TABLE 7

STATUS OF STROMATA AND PYCNIDIA AND MODE OF SPORE DISPERSAL OF FOUR *DIPLODIA* FUNGI GROWN ON PDA ADJUSTED TO DIFFERENT pH LEVELS WITH NaOH AND HCl

(Cultures were grown for 30 days at 24°C under fluorescent light, 60 ft-c, 8 to 9 hours.)

Initial pH	*D. natalensis* 157*				*D. natalensis* 213*				*B. theobromae* 44*				*P. rhodina**			
	Stromata and pycnidia			Spore dispersal	Stromata and pycnidia			Spore dispersal	Stromata and pycnidia			Spore dispersal	Stromata and pycnidia			Spore dispersal
	Loc.	Status	Hair		Loc.	Status	Hair		Loc.	Status	Hair		Loc.	Status	Hair	
3.0	S	N	H	W	S	NG	NH	D	S	N	NH	–	–	–	–	–
4.0	S	N	H	W	S	G	NH	DW	SM	N	H	–	M	N	NH	–
5.0	S	N	H	W	SM	G	NH	DW	SM	G	H	–	M	N	NH	–
6.2	SM	N	H	W	SM	N	NH	DW	SM	N	H	–	SM	N	H	W
7.1	SM	N	H	W	SM	N	NH	DW	SM	N	H	W	SM	N	H	W
8.1	SM	N	H	W	SM	N	NH	DW	SM	N	H	W	SM	N	H	W
9.0	SM	N	H	W	SM	G	NH	DW	SM	N	H	W	SM	N	H	W
10.0	SM	N	H	W	SM	G	NH	DW	M	N	NH	–	SM	N	H	W
11.0	S	N	H	W	S	NG	H	DW	SM	N	H	W	S	N	H	–

* Color and development of mycelium varied only slightly among isolates on the different media.
S, M, SM = superficial; submerged; superficial and submerged.
G, N, NG = grouped; not grouped; grouped and not grouped.
H, NH = hairy; not hairy.
D, W, DW = dry; wet; dry and in a wet matrix.
– = not observed, or only a few and not observed.

TABLE 8

STATUS OF STROMATA AND PYCNIDIA AND MODE OF SPORE DISPERSAL OF FOUR *DIPLODIA* FUNGI GROWN ON MEDIA ADJUSTED TO DIFFERENT pH LEVELS WITH DIFFERENT BUFFERS

(Cultures were grown for 30 days at 24°C under continuous fluorescent light—Grow-Lux bulbs—at 250 ft-c.)

Buffer and initial pH	*D. natalensis* 157*				*D. natalensis* 213*				*B. theobromae* 44				*P. rhodina* 86			
	Stromata and pycnidia			Spore dispersal	Stromata and pycnidia			Spore dispersal	Stromata and pycnidia			Spore dispersal	Stromata and pycnidia			Spore dispersal
	Loc.	Status	Hair		Loc.	Status	Hair		Loc.	Status	Hair		Loc.	Status	Hair	
Citrate:																
3.0	0	0	0	0	–	–	–	0	–	–	–	–	0	0	0	0
4.0	SM	GN	NH	–	SM	N	H	D	SM	N	H	D	SM	N	NH	0
5.0	SM	GN	H	–	SM	N	H	D	SM	GN	H	D	SM	N	NH	0
Phosphate:																
6.0	SM	GN	NH	–	SM	GN	H	D	SM	GN	H	D	SM	N	NH	0
7.0	–	–	–	–	SM	GN	H	D	SM	GN	H	D	SM	N	NH	0
8.0	0	0	0	0	–	–	–	–	M	N	H	–	0	0	0	0
Carbonate:																
9.2	SM	N	H	0	S	N	H	W	M	N	H	–	S	N	NH	0
10.0	SM	N	H	0	S	N	H	W	SM	GN	H	W	–	–	–	0
10.7	0	0	0	0	S	N	H	W	0	0	0	0	0	0	0	0
Acetate:																
3.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.6	0	0	0	0	SM	N	H	DW	SM	N	H	D	SM	GN	H	0
Tris:																
7.2	SM	GN	NH	–	SM	GN	H	DW	SM	GN	H	DW	SM	N	NH	0
8.2	SM	GN	NH	–	SM	GN	H	DW	SM	GN	H	DW	SM	N	NH	0
9.2	SM	GN	NH	–	SM	GN	H	D	SM	GN	H	DW	SM	N	NH	0

* The mycelium mats of these fungi varied in color from light slate gray, olive gray, and mouse gray to black. Color changes appeared to follow no pattern with respect to the medium.

S, M, SM = superficial; submerged; superficial and submerged.
G, N, GN = grouped; not grouped; grouped and not grouped.
H, NH = hairy; not hairy.
D, W, DW = dry; wet; dry and in a wet matrix.
0 = not produced.
– = not observed, or only a few and not recorded.

TABLE 9

AVERAGE NUMBER OF PYCNIDIA* AND PYCNIDIOSPORES† OF *DIPLODIA* AND RELATED GENERA AT DIFFERENT pH LEVELS

(Cultures were grown for 30 days at 24°C under continuous light—Gro-Lux type—approximately 250 ft-c.)

Buffer and initial pH	Species and isolate no.													
	Diplodia sp. 19		*B. theobromae* 44		*P. rhodina* 86		*D. natalensis* 147		*D. natalensis* 157		*D. natalensis* 213		*D. natalensis* 230	
	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores
Citrate:														
3.0	0‡	0	8	0	0	0	0	0	0	0	16	0	0	0
4.0	27	9.0	14	<1.0	46	0	21	7.5	13	<1.0	14	9.0	9	<1.0
5.0	14	12.4	11	1.6	61	0	42	16.8	19	...	26	3.2	1	2.6
Phosphate:														
6.0	32	34.5	11	4.4	41	0	16	48.2	17	1.0	16	8.0	2	4.0
7.0	10	<1.0	12	<1.0	46	0	21	2.1	31	<1.0	19	0	5	<1.0
8.0	4	0.0	8	0	0	0	49	0	0	0	2	0	0	0.0
Carbonate:														
9.2	1	1.6	3	<1.0	0	0	3	0	2	<1.0	5	6.1	8	<1.0
10.0	7	<1.0	2	1.3	0	0	11	7.1	3	0	4	1.3	1	<1.0
10.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetate:														
3.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.6	35	9.9	33	1.1	44	0	26	0	0	0	15	4.0	2	2.0
Tris:														
7.2	12	1.0	4	8.5	36	0	9	2.4	40	<1.0	7	5.4	8	3.4
8.2	17	15.6	9	1.3	62	0	16	1.1	39	0	5	7.1	8	3.9
9.0	13	13.0	4	5.2	56	0	7	<1.0	53	<1.0	4	9.1	12	7.1

* Total pycnidia per square centimeter.
† Number of spores/plate $\times 10^3$.
‡0 = not produced or not observed.

growth studies with these same fungi on different carbon and nitrogen sources (Satour, Webster, and Hewitt, 1969*a, b;* Webster, Hewitt, and Polach, 1969). Differences in display of characteristics occurred within genera, species, and individual isolates of all the fungi grown in these experiments.

EFFECTS OF TEMPERATURE

Growth and sporulation

Earlier experiments indicated that temperature as well as light may have had an effect on the production of pycnidia and pycnidiospores of these fungi.

The medium used for the temperature studies was PDA at pH around 6. Cultures were inoculated with 3-ml-diameter plugs of mycelium from the margins of four- to five-day-old cultures on water agar as described earlier.

Cultures were grown under controlled conditions in a Sheer Gillette growth chamber for 30 days before the various morphological features of the isolates were recorded.

Vegetative growth. Except for isolate 218, none of the fungi grew at 6° and 9°C for the first 48 hours, but they did show small amounts of growth during the ensuing 28 days of incubation at these low temperatures. All fungi produced initial mycelial growth during the first few days of incubation at 39°C, but continuous incubation at that temperature soon killed the mycelium.

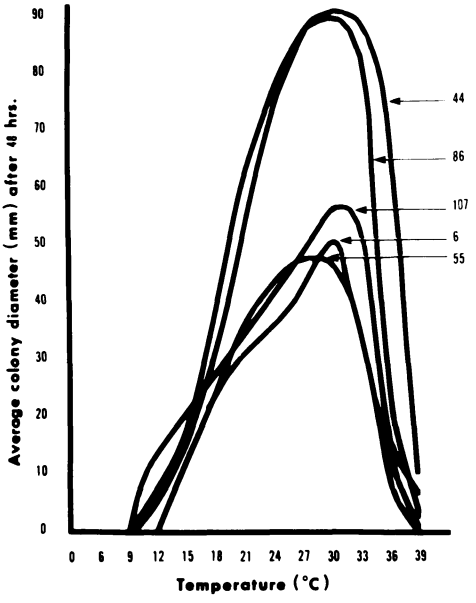

Fig. 2. Growth of different isolates of *Diplodia*-like fungi on PDA after 48 hours at different temperatures, expressed as average colony diameter of six culture plates.

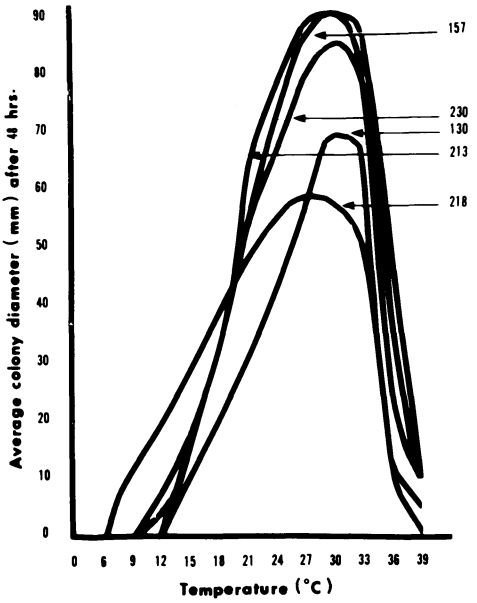

Fig. 3. Growth of different isolates of *Diplodia*-like fungi on PDA, at different temperatures, expressed as average colony diameter of six culture plates (three in each of two experiments) after 48 hours.

The amount of growth varied among cultures of isolates as well as among transfers of the same isolate grown at different temperatures.

Figures 2 and 3 show average colony diameter of several isolates after 48 hours' growth on PDA at different temperatures from 9° through 39°C.

Optimum temperatures for vegeta-

T

SPORULATION AND MORPHOLOGY OF *DIPLODIA*-LIKE FUNGI GROWN ON PDA

Isolate no.	Sporulation and morph														
	9° C							10.5° C							
	Pycnidia		Pycnidiospore					Pycnidia		Pycnidiospore					Pycnidia	
	Par.	Loc.	Col.	St.	Sep.	L.	W.	Par.	Loc.	Col.	St.	Sep.	L.	W.	Par.	Loc.
						μg	μg						μg	μg		
19	yes	MS	H	—	—	22.40	14.00	yes	MS	H	—	—	27.83	13.46	yes	S
29	no	S		n.s.	n.s.	.....	.....	n.p.	n.p.	...	n.s.	n.s.	.....	.....	no	MS
44	no	S		n.s.	n.s.	.....	.....	yes	S	LB	—	—	.....	.....	yes	MS
86	yes	MS	H	—	—	.....	.....	yes	S	LB	—	—	.....	.....	yes	MS
130	n.p.	n.p.		n.s.	n.s.	.....	.....	n.p.	n.p.	...	n.s.	n.s.	.....	.....	yes	MS
147	n.p.	n.p.		n.s.	n.s.	.....	.....	n.p.	n.p.	...	n.s.	n.s.	.....	.....	yes	MS
157	yes	MS	B	+	1	27.00	14.06	yes	MS	B	—	—	30.59	13.83	yes	MS
218	n.p.	n.p.		n.s.	n.s.	.....	.....	no	S	LB	—	—	26.36	12.74	no	S

* Par. = paraphysis; Loc. = locule; Col. = color; H = hyaline; B = brown; LB = light brown; St. = striation (+ = present; — absent) Sep. = septation; L = length; W = width; n.s. = no spores; n.p. = no pycnidia; MS = multiple- and single-loculated pycnidia; M = multiple-loculated; S = single-loculated.

tive growth for the isolates tested were: 27°C for isolates 55 and 218; 30°C for 6, 44, 86, 107, 130, 157, and 230; and 30° to 33°C for 213.

Table 10 gives results of observations on some of the useful taxonomic characters for this group of fungi as observed at various temperatures.

At the end of the 30-day incubation period, isolate 130 was the only one to have produced pycnidia and pycnidiospores at 9°C. No other isolates sporulated on plates maintained below 12°C or above 36°C.

Isolates differed in production of pycnidia and pycnidiospores in response to temperature (table 10). For example, isolate 86 sporulated only at temperatures of 15° through 24°C, while isolate 213 sporulated only at temperatures between 12° and 33°C. The location and status of pycnidia under different temperatures also varied. Pycnidia were formed either superficially and/or submerged in the media at some temperatures. Isolate 86 produced only superficial pycnidia at 15° and 18°C, and only submerged pycnidia at 21° and 27°C. Both submerged and superficial pycnidia were produced at 18° and 24°C.

All isolates that sporulated formed separate pycnidia without stromata, throughout the fruiting range. Clumps of pycnidia with and without stromata were also formed by the isolates over this range.

Spore size apparently did not vary appreciably over the temperature ranges of the experiments. Differences of very nearly the same magnitude as shown in table 10 have been observed for the same isolates in single-spore cultures grown under standard conditions.

EFFECTS OF LIGHT

Production of pycnidia and pycnidiospores

Growth conditions. Cultures were grown on PDA under varying light conditions in a Sheer Gillette growth chamber. Relative humidity was maintained at 60 per cent, and temperature was held at 25°C ± 1° over the 34-day period of incubation. Light conditions tested were as follows: (1) Continuous light for 34 days of incubation; (2)

CNIDIA AND PYCNIDIOSPORES OF EIGHT ISOLATES
FFERENT TEMPERATURES UNDER CONTINUOUS LIGHT*

mperatures of:

21° C			20° C							30° C						
Pycnidiospore			Pycnidia		Pycnidiospore					Pycnidia		Pycnidiospore				
Sep.	L.	W.	Par.	Loc.	Col.	St.	Sep.	L.	W.	Par.	Loc.	Col.	St.	Sep.	L.	W.
	μg	μg														
1	26.15	13.57	...	...	...	...	...	25.28	13.28	yes	S	B	+	1	25.66	12.94
–	26.37	17.16	yes	MS	B	–	–	24.64	17.26	...	...	...	n.s.	n.s.	.....	.....
1	26.90	13.44	yes	MS	H	–	–	25.12	14.52	...	...	...	...	...	23.69	12.67
1	26.79	13.23	yes	MS	H	–	–	26.48	13.04	n.p.	n.p.	...	n.s.	n.s.	.....	.....
1	21.72	5.00	n.p.	n.p.	...	n.s.	n.s.	.....	.....	n.p.	n.p.	...	n.s.	n.s.	.....	.....
1	22.24	10.84	n.p.	n.p.	...	n.s.	n.s.	.....	.....	n.p.	n.p.	...	n.s.	n.s.	.....	.....
1	28.34	13.47	yes	MS	B	+	1	29.42	14.98	yes	MS	B	+	1	27.39	14.16
1	24.40	13.38	yes	MS	B	–	–	23.80	12.76	yes	MS	B	–	–	24.12	9.80

continuous dark for 34 days; (3) alternating 12 hours light and 12 hours dark for 34 days; (4) 24 hours of light after four days' initial growth, followed by dark for the next 30 days of incubation; (5) two days of dark, then 15 minutes of light followed by dark for the remainder of the 34-day period.

Isolates tested. Isolates 6 and 55 were included because they are not known to have fruited under any condition in earlier experiments. Isolates 147, 157, 213, and 230 were included to give a comparison among isolates of the same species. Isolate 19 was included because it produced abundant pycnidia under normal laboratory conditions. Isolates 29 and 44 sporulated under normal laboratory conditions of day and night, and were used for comparison both among species of the so-called *Botryodiplodia* and between *B. theobromae* 44 and *D. natalensis* isolates. Isolates *D. zeae* 30 and *Physalospora rhodina* 86 appeared to be the same although the latter came to us under the Ascomycete name. Only the pycnidial form of the fungus has formed, however, in cultures in our experiments conducted thus far.

Observations and conclusions. Table 11 shows the relative production of pycnidia and pycnidiospores under the different light treatments. Except that some isolates grew more rapidly than others, variations in light conditions apparently did not have a noticeable effect on growth rate over the first four days. Isolates 6 and 55 were not induced to fruit under any conditions of light during the experiments.

In general, the isolates were observed to produce more fruiting structures under continuous light conditions. Isolates 147 and 230 fruited only when grown in constant light or in periods of alternating dark and light, whereas isolates 157 and 213 sporulated in continuous light, continuous darkness, and periods of alternating light and dark.

Pycnidia and pycnidiospores were produced more abundantly by isolates exposed to light during maturation. Although exposure to light apparently was not required for pycnidial production by most isolates, it was required for isolates 147 and 230. Thus incubation under constant light conditions in no case inhibited the production of pycnidia and pycnidiospores, and in some cases was necessary for fruiting. Perithecia were not observed in *Physa-*

TABLE 11

EFFECT OF VARIOUS LIGHT CONDITIONS ON PRODUCTION* OF PYCNIDIA AND PYCNIDIOSPORES BY ISOLATES OF *DIPLODIA* AND RELATED FUNGI

Taxon and isolate no.	Pycnidia and pycnidiospore production under:											
	Continuous light			Continuous dark			Alternating 12-hr. light 12-hr. dark		24-hr light after 4-day growth		15-min. light after 48-hr. growth	
	Exp. no.			Exp. no.			Exp. no.		Exp. no.		Exp. no.	
	1	2	3	1	2	3	1	2	1	2	1	2
Diplodia natalensis 6	−	−	−	−	−	−	−	−	−	−	−	−
D. natalensis 147	++	++	++	−	−	−	−	−	−	−	−	−
D. natalensis 230	+	+	+	−	−	−	+	+	0	0	0	0
D. natalensis 213	++	++	++	++	++	+	++	++	+	++	+	0
D. natalensis 157	++	++	++	++	++	++	++	++	++	++	++	++
Diplodia sp. 19	++	++	++	++	++	++	++	++	++	++	++	++
D. zeae 130	++	++	++	++	+	+	++	+	+	0	+	+
Botryodiplodia hypodermia 29	++	++	++	++	++	++	++	++	++	++	++	++
B. theobromae 44	++	++	++	+	+	0	+	+	+	0	0	0
Physalospora rhodina 86	++	++	++	+	+	−	+	+	+	0	+	+
Botryosphaeria ribis 55	−	−	−	−	−	−	−	−	−	−	−	−

* − = no pycnidia or pycnidiospores.
0 = pycnidia initials only.
\+ = few pycnidia and pycnidiospores.
++ = abundant pycnidia and pycnidiospores.

lospora rhodina 86 under any conditions of the varied-light experiments.

Observations on variation in morphology of pycnidia and pycnidiospores grown under the described light conditions show that the location of pycnidia with respect to the media, and production, shape, color, ornamentation, and septation of spores, etc., were similar to those observed in earlier experiments on effects of pH and temperature, and of nutrition.

EFFECTS OF VITAMINS

Growth and sporulation

It is well known that vitamins are required for growth and sporulation by some fungi but not by others, and that some fungi may compound their own supply (Lilly and Barnett, 1951; Cochrane, 1958). This section reports observations on growth and sporulation by selected isolates grown on either a minimal medium alone or on one combined with vitamins, and also on a general-purpose medium, PDA.

Materials and methods used, unless otherwise stated, were those described earlier.

BASAL MEDIUM COMPOSITION

	gm/l
Dextrose	20
Sodium nitrate	2
Potassium monophosphate	2
Potassium diphosphate	0.2
Potassium chloride	0.5
Magnesium chloride	0.5
Bacto-agar	15
Water (glass-distilled) to make 1 liter	

The pH of the medium before autoclaving was 5.5. The following vitamins, alone and in combination, were added

TABLE 12

AVERAGE COLONY DIAMETER* OF ISOLATES OF *DIPLODIA* AND *DIPLODIA*-LIKE FUNGI GROWN ON MINIMAL MEDIA WITH VITAMINS ADDED SINGLY AND IN COMBINATION

(Cultures were grown at 24°C under continuous light, in growth chambers, and measured on 4th day of incubation after inoculation.)

Vitamins added	Colony diameter (*mm*)											
	Isolate no.											
	6	19	29	44	55	86	130	147	157	213	218	230
None (control)	68	84	21	90	70	90	6	85	62	90	90	71
Vitamin A	69	88	67	90	73	90	6	55	81	90	90	80
Thiamine	73	90†	32	90	73	90	7	59	87	90	90	75
Biotin	74	88	35	90	59	90	11	87	88	90	90	83
Pyridoxine	66	87	28	90	82	90	15	76	86	87	87	75
Inositol	70	89	32	90	78	90	64	83	86	90	90	79
Vit. A + thia.	72	88	25	90	75	90	26	81	89	88	90	84
Vit. A + bio.	70	90	23	90	81	90	28	88	81	89	90	83
Vit. A + pyr.	63	73	27	90	78	90	6	65	83	90	90	78
Vit. A + inos.	73	88	34	90	74	90	12	84	88	90	90	86
Thia. + bio.	70	76	34	90	78	90	10	84	88	90	90	80
Thia. + pyr.	70	90	39	90	55	90	8	87	86	90	90	77
Thia. + inos.	77	90	33	90	69	90	17	90	90	90	90	87
Bio. + pyr.	73	69	33	90	75	90	10	88	88	90	90	86
Bio. + inos.	76	90	38	90	72	90	28	70	86	90	90	86
Pyr. + inos.	74	83	35	90	75	90	18	76	90	90	90	84

* Each measurement is the mean of two experiments of three culture plates each.
† 90 = 90 or < the fungus colony had grown to the margin of the plate.

at concentrations indicated in tables 12 and 13.

	mg/l
Vitamin A	200
Thiamine	200
Biotin	20
Pyridoxine	200
Inositol	1,000

The basal medium was steam-autoclaved at 15 lb. pressure for 15 minutes. The vitamin solutions were passed through Millipore filters and added to the basal medium after autoclaving. Solid medium was prepared by adding Bacto-agar to the basal medium before autoclaving. Petri dishes containing 15 ml of medium were inoculated with mycelium plugs as outlined under "Materials and Methods." Growth was determined by measuring the colony diameter on the fourth day of growth at 24°C under continuous light, under Gro-Lux-type fluorescent bulbs at about 250 ft-c.

Results. Table 12 shows the linear mycelial growth of 12 different isolates of *Diplodia*-like fungi grown on solid-culture medium containing different combinations of vitamins.

Most isolates grew as rapidly on the basal control medium without added vitamins as on media with a single added vitamin or with vitamins in different combinations. However, three of the isolates, *D. hypoderma* 29, *D. zeae* 130 (table 12) and *D. macrospora* 35 (table 13), grew much better on vitamins than on the control medium without vitamins. Although the growth of isolate *D. natalensis* 157 on the minimal control medium was from three to 10 times greater than that of 29, 130, and 35, it grew more rapidly on the medium with added vitamins.

Over the 30-day period of growth, all isolates, including the four mentioned above that differed in growth rate, grew on the minimal medium without exogenous sources of vitamins.

Colony type and sporulation

Isolates 6, 55, and 230 did not produce normal growth on the minimal medium nor in some vitamin combinations as compared with growth on PDA. Furthermore, they did not sporulate on the minimal medium nor in cultures containing single vitamins or any combination of vitamins.

Isolates *D. natalensis* 157, *D. zeae* 130, and *D. hypoderma* 29 sporulated only when grown on PDA or in cultures containing specific vitamins. Isolate 157 did not produce pycnidia or spores on the control plates containing minimal media. A few pycnidia were formed on plates containing various combinations of vitamin A, pyridoxine, and inositol. In no case was growth typical for that of the fungus on PDA. However, most other taxonomic characters apparently were not significantly affected.

Although isolate 130 produced pycnidia and spores abundantly on PDA, pycnidia were formed only in cultures containing biotin and in one of five different combinations containing pyridoxine.

Isolate 29, which also formed an abundance of pycnidia and spores on PDA, produced them in only four combinations: with inositol; in two combinations with biotin; and in only one combination with pyridoxine and other vitamins.

All other isolates tested grew well and sporulated on the minimal medium without added vitamins. Cultures of these isolates appeared normal when grown on the minimal medium and on media containing various combinations of vitamins, and were comparable in all respects to cultures of the isolates grown on PDA. Mycelial color, amount of aerial hyphae, hairs and setae on pycnidia, and status of pycnidia, that is, groups—separate, submerged, and not submerged in the media—all appeared unaffected on any of the culture media. Spore sizes in the various media did not differ significantly from those produced on the control plates.

TABLE 13

AVERAGE COLONY DIAMETER* OF ISOLATES OF *DIPLODIA* AND *DIPLODIA*-LIKE FUNGI GROWN ON MINIMAL MEDIA WITH VITAMINS ADDED SINGLY AND IN COMBINATION

(Cultures were grown at 24°C under continuous light, in growth chambers, and measured on 7th day of incubation after inoculation.)

Vitamins added	Colony diameter (*mm*)		
	Isolate no.		
	35	130	213
None (control)	9	90	90
Biotin	77	90	90
Thiamine	73	90	90
Pyridoxine	81	71	90
Inositol	34	90	90
Riboflavin	76	78	90
Ribo. + inos.	29	76	90
Bio. + thia.	80	90	90
Bio. + pyr.	80	61	90
Bio. + inos.	54	90	90
Bio. + ribo.	58	60	90
Bio., thia., and pyr.	66	62	90
Bio., thia., and inos.	61	90	90
Bio., thia., and ribo.	63	68	90
Bio., pyr., and inos.	54	73	90
Bio., pyr., and ribo.	67	72	90
Bio., inos., and ribo.	46	90	90
Thia. + pyr.	80	59	90
Thia. + inos.	60	90	90
Thia. + ribo.	56	52	90
Thia., pyr., and inos.	44	90	90
Thia., pyr., and ribo.	61	81	90
Thia., inos., and ribo.	35	88	90
Bio., thia., pyr., and inos.	69	87	90
Bio., thia., pyr., and ribo.	70	83	90
Bio., thia., pyr., inos., and ribo.	34	87	90
Pyr. + inos.	82	90	90
Pyr. + ribo.	64	57	90
Pyr., inos., and ribo.	54	80	90

* Each measurement is the mean of two experiments of three culture plates each.

SUMMARY AND DISCUSSION

Although growth of isolates of *Diplodia* and *Diplodia*-like fungi was altered by the pH of the growing medium, the fungi grew over a wide range of pH values. A bimodal response in growth which peaked at pH levels near 4.5 and 7.0 was common for most but not all isolates even when they were apparently of the same species. Some peaked at only one pH; others grew equally well over a wide pH range. The pH of the culture medium within ranges of good growth had little apparent influence on the mycelium color or general colony appearance. Sporulation, however, was influenced by pH, and apparently more so by the buffering system used to adjust the pH levels of the culture medium. Although the pH of the culture medium influenced production of fruiting structures and spores, it had little stabilizing effect on characteristics used in the classification of these fungi.

Temperatures ranging from 6° to 39°C had the usual expected effects. Growth of isolates increased as temperature increased, peaked at a range from 27° to 33°C, and then dropped rapidly to form a skewed curve. Temperature apparently had no influence on sporulation, which varied with the isolates, nor any significant influence on characteristics used in classification of these fungi.

In general, isolates appeared to grow better and mature more favorably in light, either continuous or cyclic, than in continuous dark, although some isolates grew and fruited normally in continuous dark. Two of the isolates, however, required light for initiation of pycnidia. Otherwise, light apparently did not greatly influence the variability of cultural or morphological characteristics normally used to differentiate the isolates.

Although biotin was apparently required for sporulation of isolates 29 and 130, the other isolates grew and sporulated on the minimal medium without biotin or other added vitamins. The presence or absence of vitamins had little or no apparent effect on sporulation or on the production and growth of classification characteristics.

Results reported in this and earlier papers have been directed mainly toward a determination of the interplay of cultural conditions and the inherent variability of these fungi, and its effect on the stability of characters used at present in classification. Initially it was hoped that the studies would lead to determination of a set of cultural conditions that would in turn lead to a standardization of characters available to all workers interested in this group of fungi. This has, in effect, been accomplished since it now appears that the main weaknesses in the classification of these fungi lie in the characters chosen and not in the cultural conditions *per se*. For example, earlier we showed (Webster, Hewitt, and Polach, 1969) that large numbers of *D. natalensis* isolates, when grown under a standard set of conditions, exhibited as much variability in morphology as has been observed in all the studies with carbon, nitrogen, C:N ratio, and other cultural conditions. Thus inherent variability in morphological characters and the influence of cultural conditions must be considered in any attempt at specific determination with members of this group.

Results of the present study confirm earlier findings (Satour, Webster, and Hewitt, 1969*a, b;* Webster, Hewitt, and Polach, 1969) that spore size, shape, and ornamentation are the most stable morphological characters in the group regardless of the cultural conditions under which the fungi are grown. The studies have further shown that the

common potato-dextrose-agar medium is satisfactory for growing these fungi for purposes of identification. We are now analyzing the effects of natural substrates on variability and also the extent of variability observable in single-spore analysis when each isolate is grown under standard conditions. At the conclusion of these studies we are confident that reliable taxonomic characters for this group will have been identified.

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ence on sporulation or characteristics used in classification. In general, isolates appeared to grow better and mature more favorably in light—either continuous or cyclic—than in continuous dark, although some did grow and fruit normally in continuous dark. Two isolates required light for initiation of pycnidia. Two required biotin for sporulation, while all others tested grew and sporulated on minimal medium without biotin or other added vitamins. Potato-dextrose-agar, available in all laboratories, is considered a satisfactory medium for growing these fungi for identification purposes.

V. Effects of Carbon:Nitrogen Ratio on Growth, Pycnidia, and Pycnidiospore Formation

Isolates of *Diplodia natalensis* and *Botryodiplodia theobromae* were grown on synthetic media containing various concentrations of carbohydrate and nitrogen sources in different carbon:nitrogen ratios to determine those ratios' effect on the growth and stability of taxonomic characters used to delimit these fungi. Growth of hyphae was favored as carbohydrate concentration increased, whereas increases of nitrogen above 1 gm sodium nitrate per liter had little effect.

Varying the carbon:nitrogen ratio of the growth media affected pigmentation, pycnidia shape and size, distribution of pycnidia, presence or absence and amount of stromata, presence or absence of hairs or setae on pycnidia, and number of pycnidiospores produced. Size and ornamentation of pycnidiospores were least affected, suggesting that these characters are least influenced by culture conditions. *D. natalensis* and *B. theobromae* are considered synonymous, since the results show that characters used previously to distinguish the fungi are significantly influenced by the media on which they are grown.

VI. Effects of Natural Substrates on Variability in Taxonomic Characters

Isolates representing nine genera and 28 species were cultured on eight natural substrates and two media. The object was to compare the effect of natural substrates on the morphology of characters currently employed to delimit genera and species of this group of fungi. Observations clearly show that valid distinctions cannot be made among most of these fungi on the basis of characters such as relationship of pycnidia to substrate, rostrate or nonrostrate pycnidia, pycnidial hairs or setae, presence or absence of stromata and distribution of pycnidia, single vs. multiloculate stromata, and paraphysis. It is proposed that pycnidiospore characters, such as gross morphology, ornamentation, and size, would be more useful for distinguishing genera and species than are those characters now employed.

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