HILGARDIA

A JOURNAL OF AGRICULTURAL SCIENCE PUBLISHED BY THE CALIFORNIA AGRICULTURAL EXPERIMENT STATION

Volume 41, Number 5 • November, 1971

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

IV. Effects of pH, Temperature, Light, and Vitamins on Certain Taxonomic Characters

W. B. Hewitt, R. K. Webster, and M. M. Satour

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

R. K. Webster, W. B. Hewitt, and M. M. Satour

VI. Effects of Natural Substrates on Variability in Taxonomic Characters

R. K. Webster and W. B. Hewitt

HILGA 41(5) 77–122 (1971)

UNIVERSITY OF CALIFORNIA DIVISION OF AGRICULTURAL SCIENCES

IV. Effects of pH, Temperature, Light, and Vitamins on Certain Taxonomic Characters

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-

(Continued inside back cover)

THE AUTHORS:

W. B. Hewitt is Professor of Plant Pathology and Plant Pathologist in the Experiment Station, Davis, and Assistant Director, Agricultural Field Stations, San Joaquin Valley Research and Extension Center, Parlier.

R. K. Webster is Associate Professor of Plant Pathology and Associate Plant Pathologist in the Experiment Station, Davis.

M. M. Satour was Postgraduate Research Plant Pathologist, Department of Plant Pathology, Davis. He is now with the Department of Plant Pathology, Ministry of Agriculture, Orman, Giza, United Arab Republic.

R. K. Webster, W. B. Hewitt, and M. M. Satour

V. Effects of Carbon:Nitrogen Ratio on Growth, Pycnidia, and Pycnidiospore Formation[1]

ABSTRACT

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. Varying the ratio 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. *D. natalensis* and *B. theobromae* are considered synonymous, since the results show that characters used previously for identification are significantly influenced by the growth media.

INTRODUCTION

In earlier studies (Satour, Webster, and Hewitt, 1969*a*, *b*) we showed that various carbon and nitrogen sources affect not only growth and sporulation but also the morphology of certain structures used to delimit genera and species in the taxon Phaeodidymous: Sphaeropsidales. The only report found by us on the effect of carbon:nitrogen (C:N) ratio on growth and sporulation in this group is by Wardlaw (1932) on *Botryodiplodia theobromae*. By varying the concentration of sucrose in a synthetic medium containing 0.2 per cent asparagine as the sole source of nitrogen, he found that growth and sporulation—and particularly production of stromata — increased as the amount of carbon was increased in the test media.

The major differences commonly used to distinguish between the genera *Diplodia* and *Botryodiplodia* (Fries, 1846–1849; Zambettakis, 1953, 1954) are concerned with distribution of pycnidia (scattered vs. caespitose) and presence or absence of a stroma. However, the validity of this separation has been challenged (Taubenhaus, 1915; Stevens, 1941; Webster, Hewitt, and Polach, 1969). At present we are evaluating the taxonomic criteria currently used under a wide range of culture conditions to determine which characters will serve for a more meaningful taxonomy of the group. This report is concerned with a comparison of the effects of carbon:nitrogen ratio on growth, sporulation, and on certain morphological characters of two very closely related and possibly synonymous nomenspecies of the group, namely, *D. natalensis* and *B. theobromae*.

[1] Submitted for publication October 26, 1970.

MATERIALS AND METHODS

Isolates studied include *Diplodia natalensis* 147 from citrus, obtained from K. T. Mickail, Egypt; *D. natalensis* 213 from Thompson Seedless grape, obtained by the authors, California; and *Botryodiplodia theobromae* 44 from Banana, ATCC 16391. Single-spore derivatives from these were maintained on potato-dextrose-agar (PDA) for use throughout the study.

Media and culture conditions

BASAL MEDIUM COMPOSITION

Potassium phosphate:	
monobasic	2 gm
dibasic	0.5 gm
Magnesium sulfate	0.5 gm
Potassium chloride	0.5 gm
Ferrous sulfate	0.001 gm
Biotin	4 ppm
Bacto-agar	15 gm
Water (glass-distilled) to make 1 liter	

The pH after autoclaving was approximately 6.0. The nitrogen source was sodium nitrate used at rates of 0, 1, 2, 3, 4, 5, and 6 gm per liter. The concentrations of sodium nitrate, glucose, and sorbose were tested in all possible combinations. Glucose and sorbose, the carbon sources tested, were used at 0, 5, 10, 15, 20, 25, and 30 gm per liter. Since our purpose was to compare the relative effects of varying the C:N ratio on these fungi, the C:N ratio is, for convenience, referred to as grams glucose, sorbose, or sodium nitrate, not as the actual carbon and nitrogen contained in the compounds.

The nitrogen and carbon were sterilized by Millipore filtration, and added to the BM after autoclaving. Media for studies in liquid culture differed only in omission of the agar. Potato dextrose agar was prepared fresh as described previously (Webster, Hewitt, and Polach, 1969).

Inoculum consisted of agar discs from the margins of colonies on BM inoculated by transferring small blocks of agar and mycelium from stock cultures on PDA. Mycelial dry weights were determined by standard methods. Methods used for making observations and measurements have been described previously (Satour, Webster, and Hewitt, 1969*a*, *b;* Webster, Hewitt, and Polach, 1969). Cultures on agar media were incubated at 24°C for 30 days in continuous light (Gro-Lux type) of approximately 250 ft-c. Liquid cultures were terminated after eight days' incubation under those conditions.

RESULTS AND OBSERVATIONS

Mycelial growth

When the three isolates were grown on BM with no added carbon or nitrogen, the radial growth was maximum, i.e., it covered the surface of the plates after four to five days. The amount of hyphal branching and aerial hyphae, however, was severely minimized. When glucose was added in different amounts, growth was favored, and branching of the hyphae and aerial hyphae increased in proportion to the amount of glucose in the medium. When sorbose was used as the carbon source, radial growth was slower (i.e., approximately 40 mm less in diameter in four days) at all concentrations tested. While radial growth appeared to be retarded with sorbose, the branching of hyphae appeared to be stimulated. The higher the concentration of sorbose (i.e., 15, 20, 25, 30 gm per liter) the lower the amount of linear growth. The level of nitrogen (i.e., 1 to 6 gm per liter) did not affect the linear growth at the various sugar

concentrations. These findings apply to all three isolates.

The effects of various C:N ratios on total growth, as determined on a dry-weight basis, are summarized in figure 1. Addition of sodium nitrate to the medium having glucose as the carbon source resulted in increases in total growth, with *B. theobromae* 44 producing more growth at all C:N concentrations than did either isolate of *D. natalensis* (147, 213). Increases in total growth did not correspond with sequential increases in sodium nitrate. The differences in total growth depicted in figure 1 indicate, as would be expected, that the addition of a nitrogen source results in increased total growth, but increasing amounts of sodium nitrate (i.e., 1 to 5 gm) in combination with various concentrations of glucose resulted in similar growth responses. A notable exception, perhaps, is that with isolates 147 and 213, 2 gm of sodium nitrate and 0 to 25 gm of glucose yielded growth surpassed only by 5 gm of sodium nitrate, while isolate 44 gave maximum growth with 2 gm of sodium nitrate and 0 to 25 gm of glucose. Results obtained in the total-growth studies, when sorbose was used as the carbon source, were similar to those observed for linear growth, in that mycelial growth was not significantly increased in any of the isolates with more than 15 gm of sorbose.

Pycnidia and pycnidiospore production

Pycnidial initials were observed as early as four days after inoculation, particularly around the inoculum. *B. theobromae* 44 was the only isolate that formed pycnidial initials on the basal medium (no added carbon or nitrogen), but sporulation was retarded when 10 gm of glucose or sorbose were added without a nitrogen source. Identical results were obtained when sodium nitrate was supplied in the absence of a carbon source. This retardation of pycnidial formation and maturation probably resulted from an imbalance

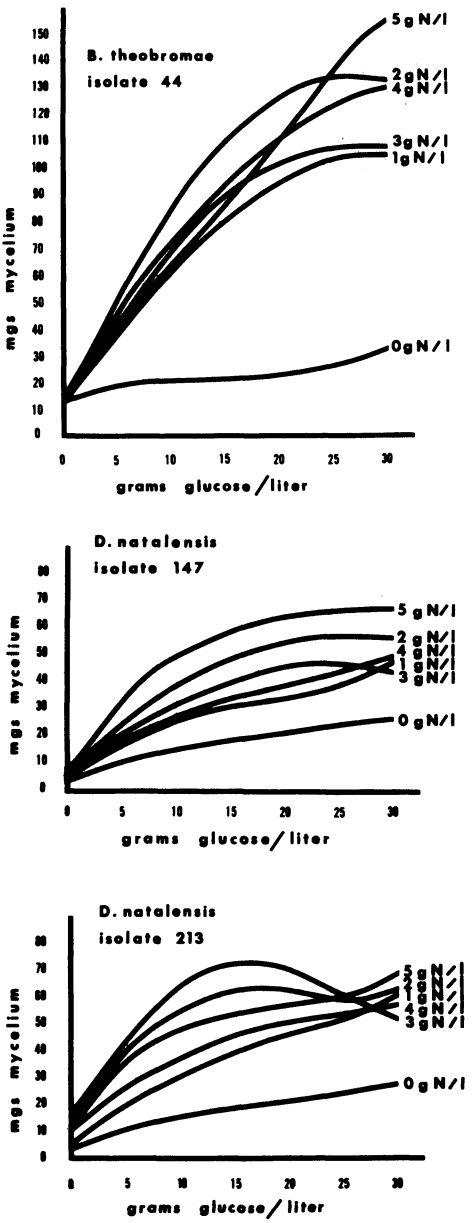

Fig. 1. Effect of varying the C:N ratio with glucose as the carbon source, on total mycelial growth (dry-weight) of *Botryodiplodia theobromae* 44 and *Diplodia natalensis* 147 and 213. Cultures were incubated for 8 days at 24°C in continuous light (Gro-Lux type), approximately 250 ft-c.

TABLE 1

AVERAGE NUMBER OF PYCNIDIA* AND PYCNIDIOSPORES† OF *DIPLODIA NATALENSIS* 147 AND 213, AND OF *BOTRYODIPLODIA THEOBROMAE* 44, GROWN ON MEDIUM B SUPPLEMENTED WITH VARIOUS AMOUNTS OF CARBON AND NITROGEN

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

Carbon:nitrogen‡ concentration (*gm/l*)	Pycnidia and pycnidiospores											
	Botryodiplodia theobromae 44				*Diplodia natalensis* 147				*Diplodia natalensis* 213			
	Carbon source				Carbon source				Carbon source			
	Glucose		Sorbose		Glucose		Sorbose		Glucose		Sorbose	
	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores
0:0 (control)	3	<1.0	2	<1.0	3	<1.0	1	<1.0	5	<1.0	3	<1.0
10:0	11	<1.0	9	2.3	21	4.7	31	6.2	5	3.8	6	13.8
10:1	38	3.7	27	13.3	34	30.7	50	30.7	14	11.6	24	35.1
10:2	27	6.5	40	18.6	34	23.4	45	37.7	23	21.9	24	45.7
10:3	25	7.1	25	17.0	38	25.1	47	42.9	20	29.3	23	52.9
10:4	20	3.0	32	18.7	45	28.0	53	37.4	21	23.7	18	53.2
10:5	33	7.9	23	15.5	31	34.1	53	35.4	22	40.7	24	50.8
10:6	21	6.4	38	21.8	43	35.0	56	42.5	25	27.8	26	47.7
0:2	1	<1.0	1	<1.0	1	<1.0	2	<1.0	2	<1.0	1	<1.0
5:2	32	3.7	18	9.1	2	10.3	54	21.8	20	10.3	27	18.6
10:2	28	5.8	25	10.2	40	....	62	45.8	26	15.0	40	31.0
15:2	32	18.7	13	21.6	32	44.5	82	71.7	12	20.5	45	51.0
20:2	20	9.3	19	32.5	28	52.2	70	73.5	14	24.4	46	54.2
25:2	29	9.0	15	21.2	23	48.3	60	73.8	6	38.7	32	48.5
30:2	4	20.7	18	27.8	10	59.1	53	57.6	18	32.4	47	111.3

* Average of 4 cm^2.
† Number of spores/plate × 10^3.
‡ Nitrogen source = sodium nitrate.

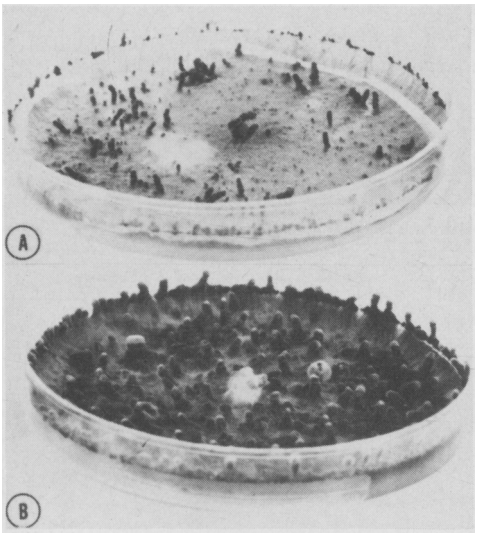

Fig. 2. Comparative effects of amount of glucose in growth medium on mycelium pigmentation, aerial hyphae, and number and dimension of stromata in *Botryodiplodia theobromae* 44. Medium A contained 5 gm glucose and 2 gm sodium nitrate. Medium B contained 30 gm glucose and 2 gm sodium nitrate. Isolate grown for 30 days at 24°C in continuous light (Gro-Lux type), approximately 250 ft-c.

in the carbon-nitrogen nutrition, which in turn favored—or at least did not retard—mycelial growth.

Glucose stimulated formation of pycnidial initials and pycnidia in all the isolates only when a source of nitrogen was present in the medium. When sorbose was the source of carbon, pycnidial initial formation varied. For example, after four days, *D. natalensis* 147 did not produce initials, while 213 did on certain amounts of sorbose, but not all. *B. theobromae* 44 did not form pycnidial initials after four days when sodium nitrate was used at 5 and 6 gm per liter and sorbose was the carbon source. The amount of nitrogen (1 to 5 gm per liter) had little effect, and no significant differences were detected between 1 and 5 gm of sodium nitrate. The qualitative differences in time required for pycnidial formation described above did not persist throughout the observation period. Observations after 30 days' incubation revealed that the three isolates (44, 147, 213) sporulated on all the C:N media combinations, including the BM. Quantitative measurements on pycnidium and pycnidiospore production on selected C:N combinations differed as shown in table 1. The optimum C:N ratio for sporulation varied among the isolates tested. For example, maximum sporulation of *B. theobromae* 44 and *D. natalensis* 147 and 213 was obtained for glucose and sorbose, respectively, at ratios of 10:1 and 10:2, 10:4 and 10:6, and 10:6 and 10:6. When the amount of carbon source varied, maximum sporulation for the three isolates was obtained on 15:2 and 10:2, 10:2 and 15:2, and 10:2 and 20:2 on glucose and sorbose, respectively. Apparently sorbose stimulated spore production, since more spores were produced with that carbon source than with glucose. The results indicate (table 1) that the optimum C:N ratio for the production of pycnidia may or may not be the same as that for production of pycnidiospores, depending on the isolate. For example, the optimum C:N ratio for pycnidia formation on glucose by *D. natalensis* 213 was 10:2, while 25:2 was the optimum for pycnidiospore production.

Effects of C:N ratio on morphological characters

Pigmentation. Since dark pigmentation of mycelium or spores is essential for the placement of fungi in this group, the effect of C:N ratios on that character were observed. On basal media, no pigment was produced by any of the isolates after 30 days' incubation. Pigment production became apparent with the addition of carbon and nitrogen (figure 2). As the carbon and nitrogen concentrations were increased, the pigment in the mycelium, fruiting bodies, and pycnidiospores became increasingly darker.

TABLE 2

AVERAGE DIAMETERS* OF PYCNIDIA AND/OR STROMATA PRODUCED BY *BOTRYODIPLODIA THEOBROMAE* 44 AND *DIPLODIA NATALENSIS* 147 AND 213, GROWN IN MEDIA SUPPLEMENTED WITH VARIOUS AMOUNTS OF CARBON AND NITROGEN

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

Carbon:nitrogen† concentrations *gm/l*	Diameter size (*μ*)					
	Botryodiplodia theobromae 44		*Diplodia natalensis* 147		*Diplodia natalensis* 213	
	Carbon source		Carbon source		Carbon source	
	Glucose	Sorbose	Glucose	Sorbose	Glucose	Sorbose
0:0	413	497	287	287	400	420
10:0	560	1,170	573	517	627	457
10:1	727	1,153	577	803	487	617
10:2	573	1,450	507	713	533	657
10:3	830	1,287	490	670	653	723
10:4	943	1,677	537	717	720	857
10:5	760	1,377	623	577	677	713
10:6	707	907	437	807	553	633
0:2	583	396	255	277	453	493
5:2	887	870	563	523	627	730
15:2	1,133	963	627	870	667	1,110
20:2	1,140	927	653	750	543	1,153
25:2	1,183	913	670	770	650	1,213
30:2	2,457	1,107	660	893	453	1,303

*Average of 30 pycnidia and/or groups of pycnidia.
† Nitrogen source = sodium nitrate.

Pycnidia shape and size. Pycnidia produced singly without stromatic tissue, in all three isolates, under all C:N regimes, were globose with short ostioles, whereas those produced in stromata varied in shape from globose-flask-shaped to irregular. Size of pycnidia was most affected by variations in C:N ratio (table 2). For example, media rich in carbohydrate favored production of large fruiting bodies, pycnidia, and/or stromata. *B. theobromae* 44 produced fruiting bodies measuring 2,457, 1,183, 887, and 583 *μ* in media containing 30, 25, 5, and 0 gm per liter of glucose, respectively. The same isolate produced fruiting bodies that measured 1,107, 913, and 396 on media containing 30, 25, and 0 gm per liter of sorbose, respectively.

Pycnidia: separate vs. grouped. All pycnidia produced on BM and medium with no added carbon source were separate. However, addition of a carbon source and variation in C:N altered the status of the pycnidia (table 3). For example, *D. natalensis* 213 produced separate pycnidia on media containing 3 gm of sodium nitrate per liter, and grouped or fused pycnidia when sodium nitrate was added at a rate of 4 gm per liter, when glucose was constant at 10 gm per liter. *B. theobromae* 44 produced grouped and separate pycnidia in all treatments with the exception of the BM containing no carbohydrate (table 3). The source of carbon influenced the status of the pycnidia. For example, when glucose was added to the media with 3 gm of sodium nitrate, *D. natalensis* 213 produced separate pycnidia only, while with sorbose added at that sodium nitrate concentration, both grouped and separate pycnidia were produced by this isolate. Concentration of sodium nitrate also interacted with the effect of these two carbon sources. For ex-

TABLE 3

MYCELIAL AND REPRODUCTIVE CHARACTERS OF *BOTRYODIPLODIA THEOBROMAE* 44 AND *DIPLODIA NATALENSIS* 147 AND 213, GROWN IN A SYNTHETIC MEDIUM SUPPLEMENTED WITH DIFFERENT AMOUNTS OF CARBON AND NITROGEN

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

Species and isolate no.	Carbon: nitrogen* concentration	Stromata and pycnidia						Spores	
		Location†		Status‡		Hairs§		Dispersal¶	
		Carbon source		Carbon source		Carbon source		Carbon source	
		Glucose	Sorbose	Glucose	Sorbose	Glucose	Sorbose	Glucose	Sorbose
	gm/l								
Botryodiplodia theobromae 44	0:0 (control)	SM	SM	N	N	NH	NH	D	D
	10:0	SM	SM	GN	GN	NH	HN	—‖	D
	10:1	SM	S	GN	GN	NH	NH	D	D
	10:2	S	S	GN	GN	NH	NH	DW	D
	10:3	S	S	GN	GN	H	H	D	D
	10:4	S	SM	GN	GN	H	NH	D	D
	10:5	S	S	GN	GN	H	H	D	D
	10:6	S	SM	GN	GN	H	NH	DW	D
	0:2	SM	SM	N	N	HN	HN	D	D
	5:2	SM	SM	GN	GN	NH	NH	D	D
	10:2	S	S	GN	GN	NH	NH	DW	D
	15:2	SM	S	GN	GN	NH	NH	DW	D
	20:2	S	S	GN	GN	NH	NH	D	D
	25:2	S	S	GN	GN	NH	NH	DW	D
	30:2	SM	S	GN	GN	NH	NH	D	D
Diplodia natalensis 147	0:0 (control)	SM	SM	N	N	NH	NH	D	D
	10:0	SM	SM	N	N	NH	NH	D	D
	10:1	S	SM	N	N	NH	NH	D	D
	10:2	S	SM	N	N	NH	NH	D	D
	10:3	S	S	N	N	NH	NH	D	D
	10:4	S	SM	N	N	NH	NH	D	D
	10:5	SM	SM	N	N	NH	NH	D	D
	10:6	S	SM	N	N	NH	NH	D	D
	0:2	SM	SM	N	N	NH	NH	D	D
	5:2	SM	SM	N	N	NH	NH	D	D
	10:2	S	SM	N	N	NH	NH	D	D
	15:2	S	SM	N	N	NH	NH	D	D
	20:2	S	SM	N	N	NH	NH	D	D
	25:2	S	SM	N	N	NH	NH	D	D
	30:2	S	S	N	N	NH	NH	D	D
D. natalensis 213	0:0 (control)	SM	SM	N	N	NH	NH	D	D
	10:0	SM	SM	GN	N	NH	NH	D	D
	10:1	SM	SM	GN	GN	NH	NH	D	D
	10:2	SM	SM	GN	GN	NH	NH	D	D
	10:3	SM	SM	N	GN	NH	NH	D	D
	10:4	SM	SM	GN	GN	NH	NH	D	D
	10:5	SM	SM	GN	GN	NH	NH	D	D
	10:6	SM	SM	GN	N	NH	NH	D	D
	0:2	SM	SM	N	N	NH	NH	D	D
	5:2	SM	SM	GN	GN	NH	NH	D	D
	10:2	SM	SM	GN	GN	NH	NH	D	D
	15:2	SM	SM	GN	GN	NH	NH	D	D
	20:2	SM	SM	GN	GN	NH	NH	D	D
	25:2	SM	SM	GN	GN	NH	NH	D	D
	30:2	SM	SM	GN	GN	NH	NH	DW	D

* Nitrogen source = sodium nitrate.
† S = superficial; SM = superficial and submerged.
‡ N = not grouped; GN = grouped and not grouped.
§ H = hairy; NH = not hairy; HN = hairy and not hairy.
¶ D = dry; W = in a wet matrix; DW = dry and in a wet matrix.
‖ — = not observed.

ample, *D. natalensis* 213 produced grouped and separate pycnidia on glucose, and separate pycnidia on sorbose (table 3). The pycnidia produced by *D. natalensis* 147 remained unaltered (separate) in all treatments in which they were formed.

Orientation of the pycnidia with respect to the substrate was influenced by the C:N ratio and was quite variable (table 3). Pycnidia produced by *B. theobromae* 44 were superficial when C:N ratios were 10:2, 10:3, 10:4, 10:5, 10:6, 20:2, and 25:2, and were both superficial and submerged at ratios of 0:0, 10:0, 0:2, 5:2, 15:2, and 30:2. *D. natalensis* 213 produced both superficial and submerged pcynidia in all C:N combinations in which it fruited.

Pycnidia: naked vs. hairy. Since the presence or absence of mycelium or setae on the pycnidia has been used to distinguish among members of this group, the effect of C:N ratio on that character was also studied (table 3). Pycnidia produced by *D. natalensis* 147 and 213 were naked, but were often surrounded by remnants of aerial hyphae. *B. theobromae* 44 produced both naked and hairy pycnidia in several of the treatments. For example, pycnidia were naked (hairless) when C:N ratios were 0:0, 10:0, 10:10, 10:2, 5:2, 10:2, 15:2, 20:2, 25:2, and 30:2 with glucose as the carbon source. Pycnidia were hairy at ratios of 10:3, 10:4, 10:5, and 10:6, and both hairy and naked in the same culture at a C:N ratio of 0:2 with no added carbon source.

Stromata. Basal media did not stimulate the production of stromata (table 3). *B. theobromae* 44 did produce stromata in all other treatments, but the size and extent depended on the amount of carbon and nitrogen added (fig. 2). In general, stromata production increased as carbohydrate concentration was increased. Both types of stromata, columnar and flat, were observed in the same culture, but the columnar type was more common. *D. natalensis* 147 did not produce stromata in any of the treatments; *D. natalensis* 213 produced stromata at certain C:N ratios (table 3). These results indicate not only that isolates vary in the production of stromata, but also that the growth media influence that character. Thus the use of stromata as a major character for distinguishing between the genera *Botryodiplodia* and *Diplodia* is questionable.

Pycnidiospores. Since Zambettakis (1953) gave high priority to size and ornamentation of pycnidiospores for distinguishing genera and species of this group, the effect of C:N ratio of the growth media on those characters was determined. Basal media with no C or N added did not favor the maturation of pycnidiospores. This was especially true for *B. theobromae* 44, in which most of the spores failed to form septa or the characteristic dark pigment. A few pycnidiospores produced by isolates 147 and 213 on media with no carbon or nitrogen added did mature and appeared normal, i.e., two-celled, elliptical, with distinct furrows in the thickened cell wall. Striation of pycnidiospores depended on maturation of the spores, septal formation, and pigment synthesis. Mature spores of all three isolates studied were typically striated regardless of the C:N ratio of the medium on which they were formed.

Sizes of the pycnidiospores, as observed in different treatments, are given in table 4. Methods for measuring have been described previously (Webster, Hewitt, and Polach, 1969). Spore size was determined from 30 spores from each of three replicate cultures. Spores produced by *B. theobromae* 44 on BM were somewhat larger (29.9×13.8 μ) than when nitrogen alone was added (17.7×8.3 μ). When both carbon and nitrogen were present in the test media, spore size of this iso-

TABLE 4

AVERAGE DIMENSIONS* OF PYCNIDIOSPORES OF *BOTRYODIPLODIA THEOBROMAE* 44 AND *DIPLODIA NATALENSIS* 147 AND 213, GROWN IN MEDIA SUPPLEMENTED WITH VARIOUS AMOUNTS OF CARBON AND NITROGEN

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

Carbon: nitrogen† concentrations (gm/l)	Dimensions of pycnidiospores (μ)											
	Botryodiplodia theobromae 44				*Diplodia natalensis* 147				*D. natalensis* 213			
	Carbon source				Carbon source				Carbon source			
	Glucose		Sorbose		Glucose		Sorbose		Glucose		Sorbose	
	Length	Width	Length	Width	Length	Width	Length	Width	Length	Width	Length	Width
0:0 (control)	29.9‡	13.8	....	....	22.1	12.0	....	....	24.3	13.3	....	....
10:0	26.8	14.4	27.0	14.5	21.8	12.1	23.6	13.3	26.0	13.7	25.3	14.0
10:1	25.0	14.1	25.6	13.9	23.1	14.5	23.2	12.6	25.2	13.7	25.5	14.4
10:2	25.8	13.6	25.4	14.1	20.8	12.3	21.9	12.3	24.8	13.5	25.1	13.2
10:3	26.2	14.0	26.0	13.8	22.4	12.2	22.1	12.5	25.2	13.3	24.6	13.3
10:4	24.2	13.0	25.5	13.4	20.2	12.0	22.6	12.3	24.7	14.2	25.1	13.4
10:5	25.4	13.6	25.2	13.5	23.2	12.7	22.0	12.2	23.5	13.4	25.5	13.4
10:6	26.8	15.2	25.3	13.7	21.1	12.2	22.0	12.3	24.9	13.8	24.6	13.5
0:2	17.7‡	8.3	....	....	21.2	11.9	....	....	25.0	13.6	....	....
5:2	25.6	13.8	25.5	14.0	21.0	12.0	22.6	12.3	25.6	13.3	24.8	13.4
10:2	25.8	13.7	29.4	17.2	21.1	12.2	21.9	12.3	24.8	13.5	24.8	13.4
15:2	25.3	14.3	25.6	14.1	21.4	12.4	21.6	12.0	24.7	13.5	25.8	13.1
20:2	26.6	12.1	25.4	12.9	23.5	12.7	21.1	12.4	21.7	13.6	25.5	13.2
25:2	24.8	13.8	26.3	13.6	21.0	12.0	20.9	12.4	23.0	13.4	24.2	13.3
30:2	26.5	14.4	26.3	13.8	21.2	11.7	21.8	12.1	24.3	13.3	26.4	13.2

* Each value represents the mean of 30 spores from each of three replications (total, 90 spores).
† Nitrogen source = sodium nitrate.
‡ Immature spores.

late was not affected significantly. The C:N ratio did not influence spore size in isolates 213 and 147. These results indicate that C:N ratio had less effect on spore size than on many of the other characters, and that when the media contained enough carbon and nitrogen to allow maturation, spore characters were very stable.

Dispersal of spores from the pycnidium varied in *B. theobromae* 44 as a result of changing the C:N ratio. Variation was not consistent, however, when the source of carbon was changed. For example, isolate 44 produced only dry spores when the C:N ratio was 0:0, 10:1, 10:3, 10:4, 10:5, 0:2, 5:2, 20:2, and 30:2 only when glucose served as the carbon source. Spores were released in both a dry and wet matrix in the same culture when C:N ratios were 10:2, 10:6, 15:2, and 25:2 with glucose only (table 3). Whether or not spores are released in a dry or wet matrix appears to depend on culture conditions, since as much variation was observed within a single isolate as between different isolates.

DISCUSSION

The results indicate that many of the morphological characters used in the past to separate fungi in this group are quite variable when the isolates are grown on media containing different concentrations and ratios of carbon and nitrogen. It is difficult to conclude which factor, carbon and nitrogen con-

centration or C:N ratio, most affected the morphology. Results indicate that nitrogen concentrations above 2 gm sodium nitrate per liter had little effect, since that and higher concentrations appeared to support normal growth and maturation throughout the observation period. On the other hand, when glucose served as the carbon source, mycelial growth—and particularly stromata production—were affected as the concentrations were increased. A comparison with media of similar C:N ratios, but with higher concentrations (10:1 vs. 20:2) (10:1 vs. 20:1), bears this out.

That increased concentration of the carbon source results in increased vegetative growth is not surprising, but the effect on the morphology of the isolates in relation to the taxonomy of this group is significant. For example, the genus *Diplodia* Fr. (Fries, 1846–1849), as formed in 1849, was characterized by scattered pycnidia, subcutaneous to erumpent, black, papillate, with one-septate, brown to black spores. Taubenhaus (1915) broadened this description to "Pycnidia black, subcutaneous to erumpent or superficial, scattered or in groups, caespitose or in a stroma; hirsute or glabrous, paraphyses present or absent, spores hyaline, one-celled when young, but one-septate, brown to dark when mature." Zambettakis (1953, 1954) maintained *Diplodia in sensu nobis,* and reserved it for species with smooth spores, paraphyses and stromata absent, and pycnidia separate, ostiolated, smooth, and fleshy-walled without necks. Goos, Cox, and Stotzky (1961) reviewed the literature and taxonomy of *B. theobromae,* and they, as have others (Shear, 1933; Stevens, 1941), observed overlapping in characters used to delimit genera. They suggested that *D. natalensis* and *B. theobromae* are identical. Our results support this conclusion.

Zambettakis (1953, 1954) placed *B. theobromae* in the genus *Lasiodiplodia* Gr. & Maubl, with *L. theobromae* (Pat.) Gr. & Maubl as the type of the genus. He erected the genus *Strianemadiplodia* Zamb., in which he placed *D. natalensis* P. Evans, and considered it a synonym of *S. frumenti.* It is interesting that *Strianemadiplodia* is characterized by furrows or striations on the mature pycnidiospores, a character common to all three of the isolates included in the present study and one that is apparently completely stable in mature spores under all of the conditions studied. We have concluded that the isolates included in the present study represent the same species. It is possible that differences observed in laboratory cultures would be more stable on natural substrates, although this was not the case in a previous study of *D. natalensis* isolates from grape (Webster, Hewitt, and Polach, 1969) and in a study by Taubenhaus (1915) in which isolates were compared on sweet potato.

The literature and present data show that much of the taxonomic confusion that has accumulated in this group is a result of the varying cultural conditions under which these fungi have been studied and a disagreement by various authors on the selection of stable morphological characters.

A suggested revision, based mainly on characters of the pycnidiospores, is being prepared and will be published elsewhere.

LITERATURE CITED

FRIES, E.
1846–1949. Elias Fries summa vegetabilium. Scandinaviae, p. 416.

GOOS, ROGER D., ELSIE A. COX, AND G. STOTZKY
1961. *Botryodiplodia theobromae* and its association with *Musa* species. Mycologia **53**:262–77.

SATOUR, M. M., R. K. WEBSTER, AND W. B. HEWITT
1969*a*. Studies on *Diplodia* and *Diplodia*-like fungi: I. Effects of carbon sources on growth in agar culture. Hilgardia **39**(22):601–29.
1969*b*. Studies on *Diplodia* and *Diplodia*-like fungi: II. Effects of nitrogen sources on growth, sporulation, and certain taxonomic characters. Hilgardia **39**(22):631–53.

SHEAR, C. L.
1933. Life histories of *Tryblidiella* species. Mycologia **25**:274–85.

STEVENS, N.
1941. Host relations in species of *Diplodia* and similar genera. Mycologia **33**:69–73.

TAUBENHAUS, J. J.
1915. The probable non-validity of the genera *Botryodiplodia, Diplodiella, Chaetodiplodia,* and *Lasiodiplodia.* Amer. Jour. Botany **2**:324–31.

WARDLAW, C. W.
1932. Observations on the pycnidium of *Botryodiplodia theobromae* Pat. Ann. Bot. **46**: 229–38.

WEBSTER, R. K., W. B. HEWITT, and F. J. POLACH
1969. Studies on *Diplodia* and *Diplodia*-like fungi. III. Variation in *Diplodia natalensis* from grape in California. Hilgardia **39**(22):655–71.

ZAMBETTAKIS, C. E.
1953. Clés dichotomiques des généres et des espèces des Phaeodidymae de la famille des Sphaeropsidaceae. Ann. Inst. Phytopath. Benaki **7**:112–60.
1954. Recherches sur la systematique des "Sphaeropsidales-Phaeodidymae." Bul. Soc. Mycol. France **70**:219–350.

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.

The journal HILGARDIA is published at irregular intervals, in volumes of about 650 to 700 pages. The number of issues per volume varies.

Single copies of any issue may be obtained free, as long as the supply lasts; please request by volume and issue number from:

Agricultural Publications
University of California
Berkeley, California 94720

The limit to nonresidents of California is 10 separate titles. The limit to California residents is 20 separate titles.

The journal will be sent regularly to libraries, schools, or institutions in one of the following ways:

1. In exchange for similar published material on research.
2. As a gift to qualified repository libraries only.
3. On a subscription basis—$7.50 a year paid in advance. All subscriptions will be started with the first number issued during a calendar year. Subscribers starting during any given year will be sent back numbers to the first of that year and will be billed for the ensuing year the following January. Make checks or money orders payable to The Regents of The University of California; send payment with order to Agricultural Publications at above address.