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

I. Effects of Carbon Sources on Certain Taxonomic Characters and on Growth in Agar Culture

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

II. Effects of Nitrogen Sources on Growth, Sporulation, and Certain Taxonomic Characters

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III. Variation in *Diplodia natalensis* from Grape in California

R. K. Webster, W. B. Hewitt, and F. J. Polach

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Isolates of *Diplodia macrospora, D. natalensis, D. zeae, Botryodiplodia hypodermia, B. theobromae, Physalospora rhodina, Botryosphaeria ribis,* and a *Sphaeropsis* sp. were grown on synthetic agar media supplemented with 23 different carbon sources used either singly or in combination: L-arabinose, D-ribose, D-xylose, D-fructose, D-galactose, D-glucose, D-mannose, L-sorbose, cellobiose, lactose, maltose, sucrose, cellulose, inulin, starch, xylan, raffinose, rhamnose, salicin, D-sorbitol, linolenic acid, palmitic acid, and pectin. Taxonomic criteria currently used to delimit these species—mycelial growth and color, stromata, pycnidial size and orientation with respect to the substrate, presence of septa, and morphology and exudation of pycnidiospores—differed, in most of the isolates, with the carbon source tested. For example, sorbose retarded mycelial growth and pigmentation but increased pycnidial production. Species on salicin developed pycnidia but not pycnidiospores. Inulin, alone or in combination with glucose, retarded hyphal pigmentation, the formation of pycnidia, and the maturation of spores. The effect of salicin was partially counteracted when it was combined with sorbose, glucose, or inulin. These results indicate the value and need for additional studies to establish standard culture conditions for use in taxonomic considerations of these fungi.

II. Effects of Nitrogen Sources on Growth, Sporulation, and Certain Taxonomic Characters

Twenty-eight nitrogen sources (20 amino acids, 2 amide derivatives of amino acids, 4 organic nitrogen, and 2 inorganic nitrogen) were used for culture of six isolates of *Diplodia natalensis* and one

Continued inside back cover

THE AUTHORS:

W. B. Hewitt is Professor of Plant Pathology and Plant Pathologist in the Experiment Station, Davis.

F. J. Polach is Research Assistant, Department of Plant Pathology, 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 is Assistant Professor of Plant Pathology and Assistant Plant Pathologist in the Experiment Station, Davis.

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

I. Effects of Carbon Sources on Certain Taxonomic Characters and on Growth in Agar Culture[1,2]

ABSTRACT

Isolates of *Diplodia macrospora, D. natalensis, D. zeae, Botryodiplodia hypodermia, B. theobromae, Physalospora rhodina, Botryosphaeria ribis,* and a *Sphaeropsis* sp. were grown on synthetic agar media supplemented with 23 carbon sources used either singly or in combination. Taxonomic criteria currently used to delimit these species differed, in most of the isolates, with the carbon sources tested. Results indicate the need for additional studies to establish standard culture conditions for use in taxonomic considerations of these fungi.

INTRODUCTION

MANY ECONOMICALLY IMPORTANT pathogens appear in the taxon Phaedidymous: Sphaeropsidales (F. L. Stevens, 1913; Grove, 1937). To identify them is difficult and sometimes impossible because various workers have used unstable taxonomic characters to delimit the species. Hewitt (unpublished data) observed that a single-spore colony of *Diplodia natalensis* P. Evans, which causes summer bunch rot of grape in California (Hewitt *et al.*, 1962), produced three forms of pycnidia when grown on sterile pea-straw agar. Those formed on the surface were hirsute (*Chaetodiplodia?*); those formed beneath the surface were beaked and glabrous (*Pellionella?*); those confined to a subepidermal position on a piece of pea straw were frequently surrounded by a stromatic tissue (*Botryodiplodia?*). When the same isolate was grown on grape canes, the pycnidia were typical of the genus *Diplodia.*

In a study of *Diplodia* and related genera, Taubenhaus (1915) reported that *Diplodia gossypii* Zim. on sweet potato showed all of the characteristics of the supposed genera *Lasiodiplodia, Chaetodiplodia, Botryodiplodia,* and *Diplodiella.* Results were similar when *Lasiodiplodia tubericola* Ell. & Ev. was inoculated on sweet potato. Taubenhaus concluded that all species placed in those genera are congeneric, and should be assigned to *Diplodia.*

Goos, Cox, and Stotzky (1961) found that some isolates of *Botryodiplodia theobromae* Pat. would produce only simple pycnidia on potato-dextrose agar, whereas other isolates of the same species grown under the same conditions formed massive columnar stromata containing several pycnidia. Voorhees

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(1942) concluded that the morphology of the pycnidium and stroma is extremely variable, but that conidial shape and size are relatively constant. N. E. Stevens (1933) concluded that, in these fungi, stromatic characters are so variable that they are entirely unreliable for purposes of classification. He found that the size of the stroma and the complexity of its spore chambers in *Diplodia mutila* (Fries) Mont. varied with the substrate. For example, a clone of a single-spore isolate was grown on twigs of raspberry and currant, and also on cornmeal agar. The stroma formed were quite different: on the thin, smooth raspberry bark the fungus formed a thin-walled, uniloculate pycnidium; on the thicker and coarser currant bark, it formed a thick-walled, multiloculate pycnidium. On cornmeal agar the stromata were loose-textured and thick. Stevens also noted that humidity influenced the form of the pycnidia produced.

Many species undoubtedly are erected on the basis of behavior on various hosts (N. E. Stevens, 1941; Wehmyer, 1964). Stevens demonstrated that many species are facultative parasites with a wide host range and that their morphology could be easily modified by the nature of the host tissue itself. Thus, a great many names have probably been applied to forms of the same omnivorous species on different substrates. Wehmeyer, while regretting the necessity for erecting five new species of *Diplodia* on the basis of their occurrence on new hosts, suggested that, until overlapping characters can be sorted out and good differential ones established, there is no alternative.

The only intensive taxonomic revision of the group was done by Zambettakis (1953, 1954*a*, *b*), in which 1,524 species and forms representing 40 genera were reduced to 248 species and forms in 23 genera. Fifteen genera were retained (seven in *sensu nobis*, two emended, and six unaltered), 20 genera were reduced to synonymy, and eight new genera were erected. Zambettakis examined over 2,000 herbarium specimens but studied only 53 cultures representing 11 genera and 49 species. Thus, his studies failed to establish the stability of characters and their natural range of variation.

In our laboratory, both the range of variation in *Diplodia natalensis* P. Evans islated from grapes in California, and *Diplodia* and *Diplodia*-like fungi from other areas are being studied. We are also concerned with the possible influence of nutrition and various environmental factors on morphological characters used in identification. Our ultimate objective is the development of a system of classification for the group based on a more stable and standard set of morphological characters.

The present paper deals with the effects of different carbon sources on growth, pycnidial formation, and certain morphological characters of selected fungi in agar culture. No attempts were made to explore the physiology of sugar utilization. Results of further work on the effect of various carbon sources on growth of the isolates in liquid culture will be reported in a subsequent paper.

REVIEW OF LITERATURE

Only a few studies have been made on the role of carbon in the reproduction and alteration of morphological characters in this group of fungi.

Kinsel (1937) found that, under the conditions of her experiments, *Diplodia macrospora* Earle could not utilize monosaccharides as a source of carbon, but grew readily when supplied with di- or polysaccharides. In contrast, *D. zeae*

(Schw.) Lev. grew well on media containing monosaccharides, as well as on solutions containing more complex carbohydrates.

N. E. Stevens and Larch (1939) found that the characteristic noted by Kinsel (1937) was common to 24 isolates of *D. macrospora* from the southern United States and Argentina. Margolin (1940) has indicated that *D. macrospora* makes only sparse growth when supplied with either sucrose or glucose in the absence of biotin.

Wilson (1942) found that *D. macrospora* utilized complex carbohydrates in many instances, but only because they contained biotin as an impurity. When inulin, maltose, or sucrose was used alone as the sole source of carbon, *D. macrospora* made little growth with pure sources, but showed luxuriant growth of mycelia when commercial grades were used.

Brown (1957) tested six sugars as sources of carbon for *Botryosphaeria ribis* Gross. & Dug. Mycelial growth for the various sources was, in descending order: fructose, arabinose, glucose, sucrose, galactose, and mannitol.

Extensive work on the utilization of carbon by several fungi was reported by Lilly and Barnett (1953), but their study did not include any member of the group under investigation here. However, a *Sphaeropsis* sp. grew well on all carbohydrates tested except L-sorbose, D-xylose, and L-lactose.

Drake and Moore (1966) recently found that *Botryosphaeria ribis* grown in a semisynthetic liquid medium at pH4 made excellent growth when the sole source of carbon was maltose, sucrose, D-glucose, D-fructose, amylopectin, dextrin, sodium polypectate, galacturonic acid, polygalacturonic acid, pectin, or L-malic acid. Starch, cellulose, and ascorbic acid supported intermediate growth; citric acid supported least growth.

Only Wardlaw (1932) considered the effect of carbon concentration on mycelial growth, pigmentation, and morphology of the pycnidia and stromata. Sucrose supplied in a synthetic medium at a concentration of 0.5 per cent did not support good mycelial growth, and the mycelia were pale gray. Stromata and pycnidia, when present, were very small and few in number. Mycelia with dark green color, and larger pycnidia and stromata were developed abundantly when sucrose was supplied at a concentration of 1 to 12 per cent. At higher concentrations, stromata formed more slowly and tended to be overgrown by a dense mat of aerial hyphae. Results were similar with starch.

MATERIALS AND METHODS

Sources of cultures

Table 1 lists the species included in this study, their hosts, and sources. These particular isolates were chosen from among several of the same species for various reasons. Isolates 6 and 107 of *Diplodia natalensis* and isolate 55 of *Botryosphaeria ribis* were selected because they failed to produce pycnidia and/or perithecia on potato-dextrose-agar (PDA), V-8 juice, or Czapek agar. *Physalospora rhodina* (Berk. & Curt.) Cooke 86 produced only a few pycnidia and no perithecia on those media. Isolates 157, 213, and 230 of *D. natalensis*, from grape in California, showed cultural variation. *Sphaeropsis* sp. 218 was isolated from a grape cane also infected with *D. natalensis*. Isolates 29 and 44 of *Botryodiplodia* were included for comparison with *D. natalensis* and other genera. Isolate 147 (*D. natalensis* from citrus) was selected because of its source and its potential for producing abun-

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
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.

dant pycnidia. *D. zeae* 130 and *D. macrospora* 35 were chosen for comparison with results reported previously. Isolate 19 of *Diplodia* sp. was tested because of its host, origin, and production of numerous pycnidia on several media.

Culture media

Two synthetic media were used as controls:

COMPOSITION	MEDIUM A *gm/l*	MEDIUM B *gm/l*
Sodium nitrate	2.0	2.0
Potassium phosphate (monobasic)	–	2.0
Potassium phosphate (dibasic)	1.0	0.5
Potassium chloride	0.5	0.5
Magnesium sulfate	0.5	0.5
Ferrous sulfate	0.001	0.001
Biotin	–	4.0 (ppm)
Bacto agar	15.0	15.0
Glass-distilled water to make 1 liter		
pH after autoclaving	5.5	6.0

The media were prepared in lots of at least 10 liters to minimize differences between batches. The pH of medium A was adjusted with HCl and KOH before autoclaving, and was checked at the time plates were poured. Media were sterilized for 15 minutes at 121°C. Plates containing approximately 20 ml were inoculated with small discs (3 mm diameter) of the fungus culture grown on water agar, and were incubated for 35 days at 24°C.

In the experiments with medium A, plates were exposed for 8 to 9 hours daily to a fluorescent light, daylight type, of approximately 60 ft-c. Early in the experimental period we observed that light stimulates pycnidial formation. Consequently in subsequent experiments with medium B, the cultures

were exposed to a continuous light (Gro-Lux type) of approximately 250 ft-c.

Carbon sources

The following compounds were added to the control media as sources of carbon for the *Diplodia*-like fungi: L-arabinose, D-ribose, D-xylose, D-fructose, D-galactose, D-glucose, D-mannose, L-sorbose, cellobiose, lactose, maltose, sucrose, cellulose, inulin, starch, xylan, raffinose, rhamnose, salicin, D-sorbitol, linolenic acid, sodium pyruvate, palmitic acid, and pectin. Our purpose was to determine the effect of different carbon sources on the morphology of the various structures, rather than to make a detailed study of the utilization of the compounds. We worked with unusually large amounts of media, and chose methods that could be used under almost any laboratory conditions.

The carbon sources were used at a rate of 10 gm per liter of medium except for palmitic acid (3.5 gm) and sodium pyruvate (6 gm). When two carbon sources were mixed, 5 gm of each were used per liter. All compounds were added before sterilization. No significant change in color of the media was observed after autoclaving. All chemicals were of analytical reagent grade to meet American Chemical Society standards except for commercial grades of anhydrous glucose and sucrose.

The entire culture on a given plate was added to 80 ml of water and mixed for 3 to 5 minutes in a Waring Blendor. Final volume was adjusted to 100 ml. The pycnidiospores were then counted in a hemacytometer.

RESULTS

Vegetative growth

Determining growth of a fungus by measuring colony diameters on an agar medium has apparent limitations when compared with the more approved method of dry-weight determinations. However, the agar medium was essential to the principal objectives of evaluating effects of different carbon compounds on morphology of fruiting structures.

Tables 2, 3, and 4 list the mycelial growth, as average colony diameters, produced by isolates of species of *Diplodia* and related genera on the various media. The same agar cultures were used to obtain data on sporulation and morphological characters in addition to vegetative growth. Although the mycelium was generally submerged or appressed to the surface, the nine isolates shown in table 3 grew moderately well on control medium B without added sources of carbon. This growth may have resulted from utilization of material(s) from the agar, and further studies are being made of growth on media with no added carbon compounds. Preliminary results obtained with liquid culture suggest that these isolates are capable of growth when carbon-containing compounds are omitted from the media.

Effect of single carbon sources

Medium A. In a preliminary test (table 2), medium A was supplemented, individually, with 15 carbon sources. Various isolates utilized some carbon compounds better than others, and growth of isolates of the same species varied. For example, sucrose, raffinose, starch, and glucose supported greater initial growth of *Botryodiplodia theobromae* (isolate 44) than did the rest of the carbon sources. Three isolates of *Diplodia natalensis* (157, 213, and 230) varied in growth on certain carbon sources. Isolate 157 grew most rapidly, on maltose. Isolates 157 and 230 utilized

TABLE 2

AVERAGE COLONY DIAMETERS (LINEAR GROWTH) OF ISOLATES OF *DIPLODIA* AND RELATED GENERA ON MEDIUM A SUPPLEMENTED WITH VARIOUS CARBON SOURCES

(Cultures incubated at 24°C for four days)

Carbon source	Colony diameters									
	Isolate no.									
	6	44	55	86	107	130	157	213	218	230
	mm	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
Medium A (control)	36*	32	30	46	30	20	46	42	36	53
Medium A plus:										
D-ribose	43	43	33	46	37	25	57	52	39	42
X-xylose	32	42	24	42	34	25	37	37	12	55
D-fructose	45	44	31	53	53	28	51	49	19	77
D-galactose	41	49	33	59	49	7	58	59	32	65
D-glucose	45	52	42	67	43	7	72	75	29	76
L-sorbose	14	20	8	39	10	9	23	22	7	63
Cellobiose	38	44	38	64	37	15	82	47	30	79
Lactose	42	49	38	57	32	23	62	47	40	32
Maltose	42	51	39	55	45	20	86	62	20	70
Sucrose	33	63	39	46	37	23	71	61	28	75
Inulin	35	39	34	40	35	13	48	45	38	43
Starch	42	56	48	58	42	25	66	55	47	75
Raffinose	35	58	36	63	38	9	69	59	33	65
Rhamnose	31	44	35	54	34	27	77	61	34	56
Salicin	28	44	30	51	24	17	67	48	33	48

* Average of three plates.

cellobiose and maltose more readily than did isolate 213, and all three made about the same growth on glucose. L-sorbose inhibited growth of all isolates except 230, which made more growth than on the medium with no added carbon.

Medium B. Table 3 shows the colony diameters on nine isolates of *Diplodia* after four days of growth on medium B either with or without any one of 23 carbon compounds. All nine isolates grew moderately well on the meduim with no added carbon. When growth either failed or was retarded, we assumed that the added carbon was the growth inhibitor. The isolates varied in their response to carbon sources. For example, none had shown any growth on linolenic acid by the fourth day. (Isolates 35, 44, 147, and 213 on this medium, however, had covered the plates by the thirtieth day.) Isolates 147, 157, 203, and 230 of *D. natalensis* varied in their utilization of cellulose, and in an experiment not reported here, isolate 107 of the same species failed to utilize that compound even after 35 days. L-sorbose also inhibited growth of all isolates on medium B. Growth on this compound was less than the mean growth with almost all other carbon sources.

Mean growth on the 23 compounds was similar for isolates 19, 44, 147, 157, and 213. *D. macrospora* 19 made the weakest growth, with a mean colony diameter of 18.5 mm (range, 4 to 29 mm).

Since all nine isolates in table 3 grew moderately well on the control medium B, it must be assumed that in instances where an isolate failed to grow on the medium plus a carbon-source compound the latter was actually inhibitory to the isolate. Thus, L-sorbose added to medium B (table 3) inhibited growth of all isolates. The compound completely inhibited the growth of isolate 29 and

TABLE 3

AVERAGE COLONY DIAMETERS (LINEAR GROWTH) OF ISOLATES OF *DIPLODIA* AND RELATED GENERA ON MEDIUM B SUPPLEMENTED WITH VARIOUS CARBON SOURCES

(Cultures incubated at 24°C for four days)

Carbon source	Colony diameters								
	Isolate no.								
	19	29	35	44	130	147	157	213	230
	mm	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
Medium B (control)	63	40	18	85	51	83	54	83	39
Medium B plus:									
L-arabinose	86*	43	26	90	50	90	87	90	59
D-ribose	67	13	20	84	49	90	83	85	49
D-xylose	57	12	15	73	40	71	78	76	31
D-fructose	84	43	22	60	48	90	90	96	76
D-galactose	89	46	22	90	51	90	89	90	66
D-glucose	90	53	18	90	58	90	90	90	73
D-mannose	69	17	20	85	46	90	79	87	51
L-sorbose	35	0	9	46	8	37	38	43	26
Cellobiose	84	45	20	90	42	89	88	90	52
Lactose	70	45	12	89	53	90	84	83	50
Maltose	90	40	27	90	70	90	90	90	71
Sucrose	90	39	24	90	45	90	90	90	86
Cellulose	75	30	12	75	35	85	85	85	40
Inulin	68	29	22	83	33	88	78	88	54
Starch	85	49	16	84	49	90	90	87	83
Xylan	90	45	20	90	60	90	90	90	90
Raffinose	90	45	21	90	57	90	90	90	67
Rhamnose	82	43	18	90	48	90	90	90	51
Salicin	88	50	10	90	57	90	90	90	63
D-sorbitol	57	30	29	85	34	85	84	85	71
Linolenic acid	0	0	0	0	0	0	0	0	0
Palmitic acid	—	—	16	78	53	82	81	—	7
Pectin	—	—	27	85	76	85	85	85	85
Mean†	73.14	34.4	18.5	79.7	46.4	81.9	79.3	81.9	55.8

* Average of three plates.
† Mean growth on all sources of carbon.

nearly so that of isolates 35 and 130. Growth was also less on L-sorbose than the mean growth of isolates on almost all carbon sources. Added to medium A (table 2), L-sorbose inhibited growth of all isolates except 230. This fungus grew more on medium A plus L-sorbose than on control medium A. Linolenic acid was inhibitory to all isolates (table 3).

Effect of mixed carbon sources

Fungi tested were *B. theobromae* 44 and two isolates of *D. natalensis*, 157 and 213. Inulin, salicin, sorbose, and glucose were added to control agar media B before sterilization, singly and in various combinations (table 4). Growth of the three isolates was least rapid on sorbose. Furthermore, growth of isolates was only slightly faster on combinations of sorbose plus either salicin or inulin than on sorbose alone, and much slower than on salicin or inulin alone. Except for isolate 44, which grew slowly on salicin plus inulin, the isolates grew equally well on salicin plus inulin or glucose, and on inulin plus glucose. Sorbose apparently retarded vegetative growth of the isolates, and this effect was retained when sorbose was combined with carbon com-

TABLE 4

AVERAGE COLONY DIAMETERS (LINEAR GROWTH) OF *DIPLODIA NATALENSIS* AND *BOTRYODIPLODIA THEOBROMAE* GROWN IN MEDIUM B SUPPLEMENTED WITH FOUR CARBON SOURCES EITHER SINGLY OR IN PAIRS

(Cultures incubated at 24°C for four days)

Taxon and isolate no.	Colony diameters								
	Carbon sources								
	Salicin	Inulin	Sorbose	Glucose	Salicin + inulin	Salicin + sorbose	Salicin + glucose	Inulin + sorbose	Inulin + glucose
	mm	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
Botryodiplodia theobromae 44	72*	77	33	90	41	42	85	49	77
Diplodia natalensis 157	85	85	41	85	85	41	85	42	83
D. natalensis 213	73	80	32	85	78	40	85	52	77

* Average of three plates.

pounds that favored rapid growth when supplied alone.

Pycnidia, stromata, and pycnidiospore formation

Species of *Diplodia* and related genera varied in the formation of stromata, pycnidia, and pycnidiospores on the different carbon compounds (table 5; figs. 1 to 8). On control agar medium B, all isolates grew fairly well (table 3) and all except isolates 29 and 130 produced a few pycnidia and pycnidiospores (table 5; fig. 6). L-sorbose, which retarded growth of all isolates, stimulated pycnidia and pycnidiospore formation in some isolates. Salicin appeared to stimulate production of pycnidia (table 5), but not of pycnidiospores. Carbon sources that favored mycelial growth did not necessarily support production of pycnidia and pycnidiospores. For instance, certain isolates (6, 35, 55, 107, and 230) did not fruit on any of the media tested.

Six of the seven isolates previously known to produce pycnidia fruited well on media containing D-glucose, D-galactose, D-fructose, D-mannose, and L-sorbose, except isolate 29, which did not grow on L-sorbose even after 30 days (fig. 6). *Botryodiplodia hypodermia* 29 did not form pycnida and pycnidiospores on media supplemented singly with cellobiose, salicin, linolenic acid, potassium oxalate, sodium acetate, or sodium citrate. *B. theobromae* 44 did not fruit when lactose, potassium oxalate, or sodium citrate was the sole source of carbon (fig. 5). Only *D. zeae* 130 fruited on lactose.

With one exception, all isolates formed pycnidial stromata on medium B with salicin but failed to form pycnidiospores on these different media. *Diplodia zeae* 130 formed a few spores.

Neither salicin nor inulin alone supported good pycnidial formation, and salicin inhibited formation of pycnidiospores on *Botryodiplodia theobromae* 44 and *Diplodia natalensis* 157 and 213 (table 5). When the two carbon sources were combined, however, pycnidia and pycnidiospores of those isolates were formed in greater quantities than with inulin alone. Thus, inulin counteracted the effect of salicin on pycnidiospore production (table 6; figs. 7 and 8). Salicin plus sorbose also increased the production of pycnidial stromata and pycnidiospores of the three isolates. Salicin plus glucose slightly reduced the formation of pycnidial stromata but increased spore formation in isolates 44

TABLE 5

AVERAGE NUMBER OF PYCNIDIAL STROMATA AND PYCNIDIOSPORES PRODUCED BY ISOLATES OF *DIPLODIA* AND RELATED GENERA ON MEDIUM B SUPPLEMENTED WITH VARIOUS CARBON SOURCES

(Cultures incubated at 24°C for 30 days)

Carbon source	No. of pycnidia (per cm³) and pycnidiospores (per plate × 10³)													
	Species and isolate number													
	Diplodia sp. 19		*B. hypodermia* 29		*B. theobromae* 44		*Diplodia zeae* 130		*D. natalensis* 147		*D. natalensis* 157		*D. natalensis* 213	
	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores	Pycnidia	Pycnidio-spores
Medium B (control)	<1	5.0	0	0.0	2	<1.0	—*	8.3	3	<1.0	4	<1.0	2	<1.0
Medium B plus:														
L-arabinose	20	256.0	83	11.7	8	76.7	—	63.3	28	278.0	8	28.3	17	174.0
D-ribose	11	35.0	0	0.0	2	5.8	—	252.0	11	45.0	4	15.8	4	37.5
Xylose	17	376.0	79	11.7	5	91.6	—	566.0	13	—	10	107.5	4	264.0
D-fructose	28	356.0	111	0.0	8	90.0	—	134.0	21	207.0	10	20.0	16	174.0
D-galactose	16	255.0	56	46.0	3	60.8	—	137.0	11	265.0	6	34.2	7	119.2
D-glucose	27	315.0	49	0.0	5	35.8	—	51.6	32	220.0	2	29.4	16	216.6
D-mannose	19	408.0	67	—	6	81.6	—	166.0	19	418.0	17	65.0	6	370.7
L-sorbose	41	—	0	0.0	14	177.0	—	704.0	64	413.0	23	126.5	33	425.8
Cellobiose	18	286.0	0	0.0	5	70.8	—	423.0	21	372.0	11	36.5	15	264.0
Lactose	0	0.0	0	0.0	0	0.0	—	266.0	0	0.0	0	0.0	0	0.0
Maltose	26	256.0	6	0.0	5	38.4	—	165.0	33	146.0	15	13.3	11	115.0
Sucrose	16	238.0	22	0.0	8	69.2	—	1009.0	7	200.0	6	24.2	25	210.0
Cellulose	13	275.0	8	30.0	1	83.4	—	1019.0	14	214.0	3	60.8	4	199.9
Inulin	4	12.5	5	2.5	2	2.0	—	74.2	1	2.5	2	0.0	2	12.0
Starch	22	358.0	38	10.0	8	5.0	—	1440.0	28	321.0	18	34.2	14	150.0
Xylan	16	212.0	34	5.0	8	92.5	—	118.0	17	286.0	16	37.5	26	120.0
Raffinose	24	271.0	5	0.0	5	71.5	—	393.0	19	212.5	19	23.4	20	22.0
Rhamnose	17	228.0	67	9.2	2	32.5	—	421.0	15	136.0	34	8.3	4	197.5
Salicin	51	0.0	0	0.0	13	0.0	—	1.7	38	1.0	31	0.0	25	0.0
D-sorbitol	—	381.0	46	0.0	15	87.5	—	744.0	53	393.0	—	82.5	50	210.0
Linolenic acid	—	0.0	0	0.0	17	27.0	—	0.0	92	244.0	—	0.0	17	30.8
Palmitic acid	—	—	—	—	32	10.0	—	255.0	53	127.0	—	19.2	—	—
Pectin	—	—	—	—	28	38.4	—	97.0	53	234.0	—	34.2	42	116.0

* Not measured.

TABLE 6

AVERAGE NUMBER OF PYCNIDIA* AND PYCNIDIOSPORES† PRODUCED BY ISOLATES OF *BOTRYODIPLODIA THEOBROMAE* AND *DIPLODIA NATALENSIS* ON MEDIUM B SUPPLEMENTED WITH SALICIN, INULIN, SORBOSE, AND GLUCOSE EITHER SINGLY OR IN COMBINATION

(Cultures incubated at 24°C for 30 days)

Carbon source	Species and isolate no.					
	B. theobromae 44		*D. natalensis* 157		*D. natalensis* 213	
	Pycnidia	Pycnidiospores	Pycnidia	Pycnidiospores	Pycnidia	Pycnidiospores
Salicin	11	0.0	12	0.0	7	0.0
Inulin	4	2.3	3	20.0	1	5.7
Sorbose	26	113.0	50	428.0	37	545.0
Glucose	42	88.5	84	55.7	30	190.0
Salicin + inulin	4	39.6	9	42.5	17	53.1
Salicin + sorbose	53	121.0	67	100.0	36	157.4
Salicin + glucose	28	318.0	18	49.2	33	307.5
Inulin + sorbose	28	75.0	78	40.0	26	315.0
Inulin + glucose	1	2.9	3	5.0	2	3.5

* Pycnidia per square centimeter; average of 9 cm^2 from each of three culture plates.
† Number of spores/plate $\times 10^3$.

and 213. On the other hand, inulin plus glucose greatly reduced fruiting of the three isolates, and inulin plus sorbose decreased the formation of pycnidial stromata and pycnidiospores by isolates 44 and 157 but not isolate 213 (table 6; figs. 7 and 8). Carbon sources obviously have a strong influence on development and productivity of fruiting structures in these fungi.

Variation in other taxonomic characters

Early in the experiments it became apparent that different carbon-containing compounds would have a marked influence on hyphal pigmentation. Later, the compounds were found to influence formation of pycnidia and the morphology of fruiting structures.

Mycelium. Pigmentation of the hyphae was one of the most interesting character changes noted among isolates grown on different sources of carbon. In the first experiment, with medium A, the same isolate on different carbon sources produced pigmented hyphae varying from white to almost black. This effect was first thought to be the result of change in pH during growth, but results were almost the same when the experiment was repeated with the buffered medium B.

The hyphae of isolate 230 were grayish-olive on glucose, cream-buff on mannose, deep olive-buff on fructose, olive-gray on xylose and arabinose, and drab on salicin. None of the species tested was stable with respect to hyphal pigmentation. On mannose, isolate 230 diffused a red pigment into the agar and on the aerial hyphae. When two carbon sources were combined in the medium, the hyphal pigmentation was either absent or intermediate or did not change (figs. 7 and 8). Inulin, either alone or combined with glucose, inhibited hyphal pigmentation in all isolates tested.

Pycnidia. The pycnidia of *D. natalensis* are globose to subglobose; pycnidia of *B. theobromae* range from round or ovoid, with a short, straight neck, to typical flask-shaped structures (Goos, Cox, and Stotzky, 1961; Wardlaw, 1932).

We found that the carbon source influenced the shape of the pycnidia. For example, in the control medium B and

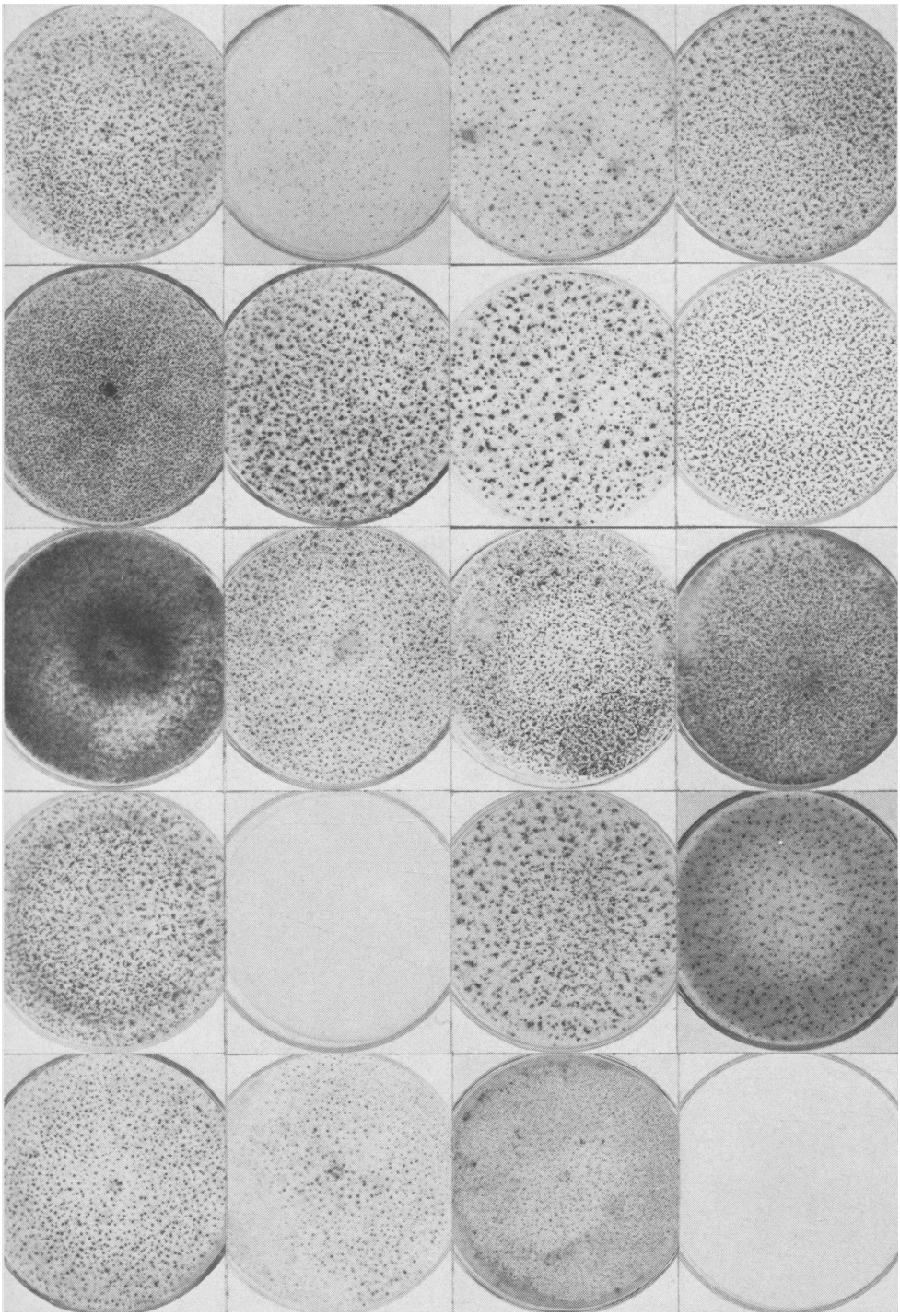

Fig. 1. *Diplodia natalensis* 147, grown for 30 days on basal medium (control) and on various carbon sources. Left to right, top to bottom: glucose, D-ribose, D-galactose, D-fructose, L-sorbose, D-mannose, D-xylose, L-arabinose, lactose, cellobiose, maltose, sucrose, starch, inulin, xylan, cellulose, raffinose, rhamnose, salicin, and basal medium.

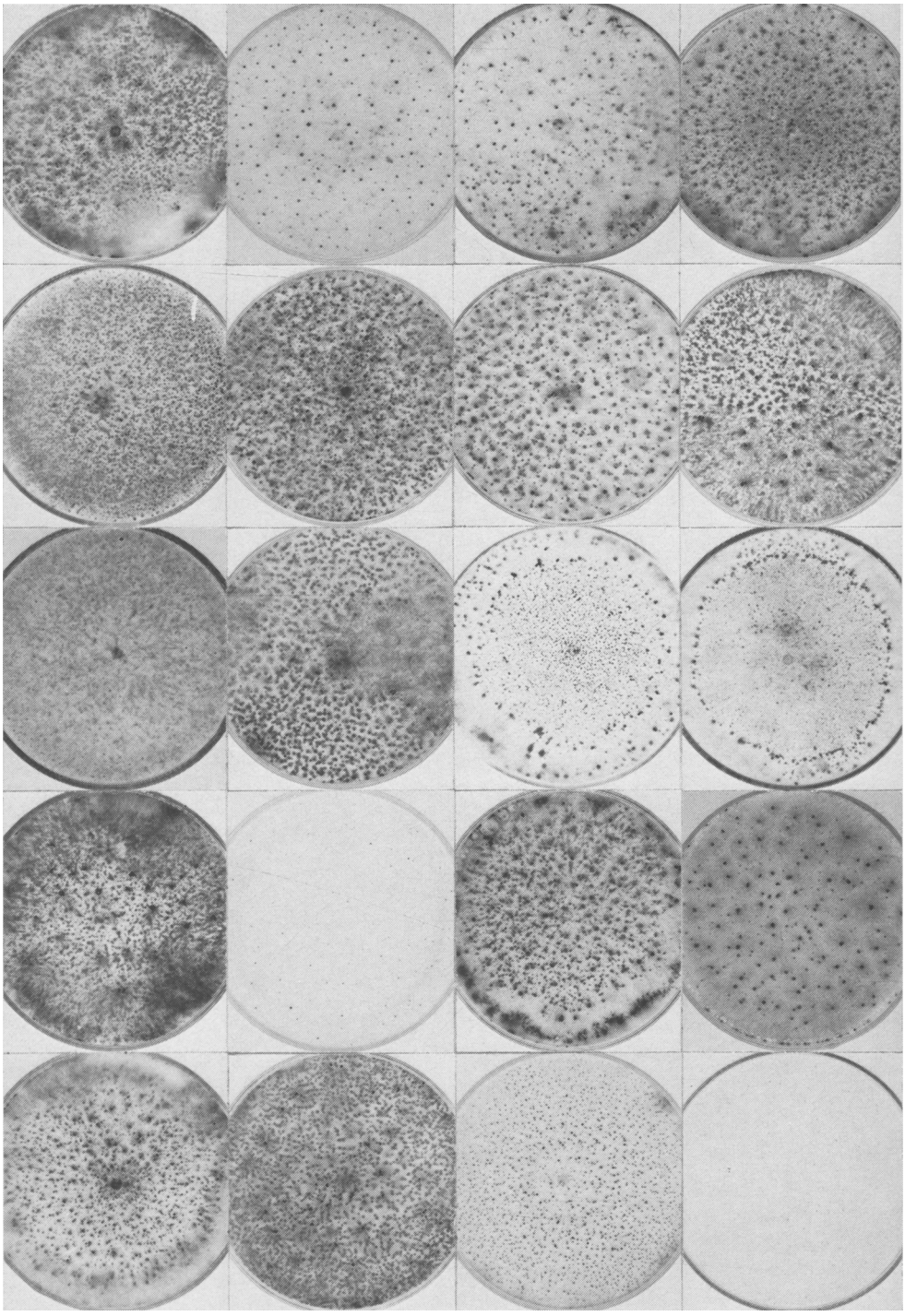

Fig. 2. *Diplodia natalensis* 157, grown for 30 days on basal medium (control) and on various carbon sources. Left to right, top to bottom: glucose, D-ribose, D-galactose, D-fructose, L-sorbose, D-mannose, D-xylose, L-arabinose, lactose, cellobiose, maltose, sucrose, starch, inulin, xylan, cellulose, raffinose, rhamnose, salicin, and basal medium.

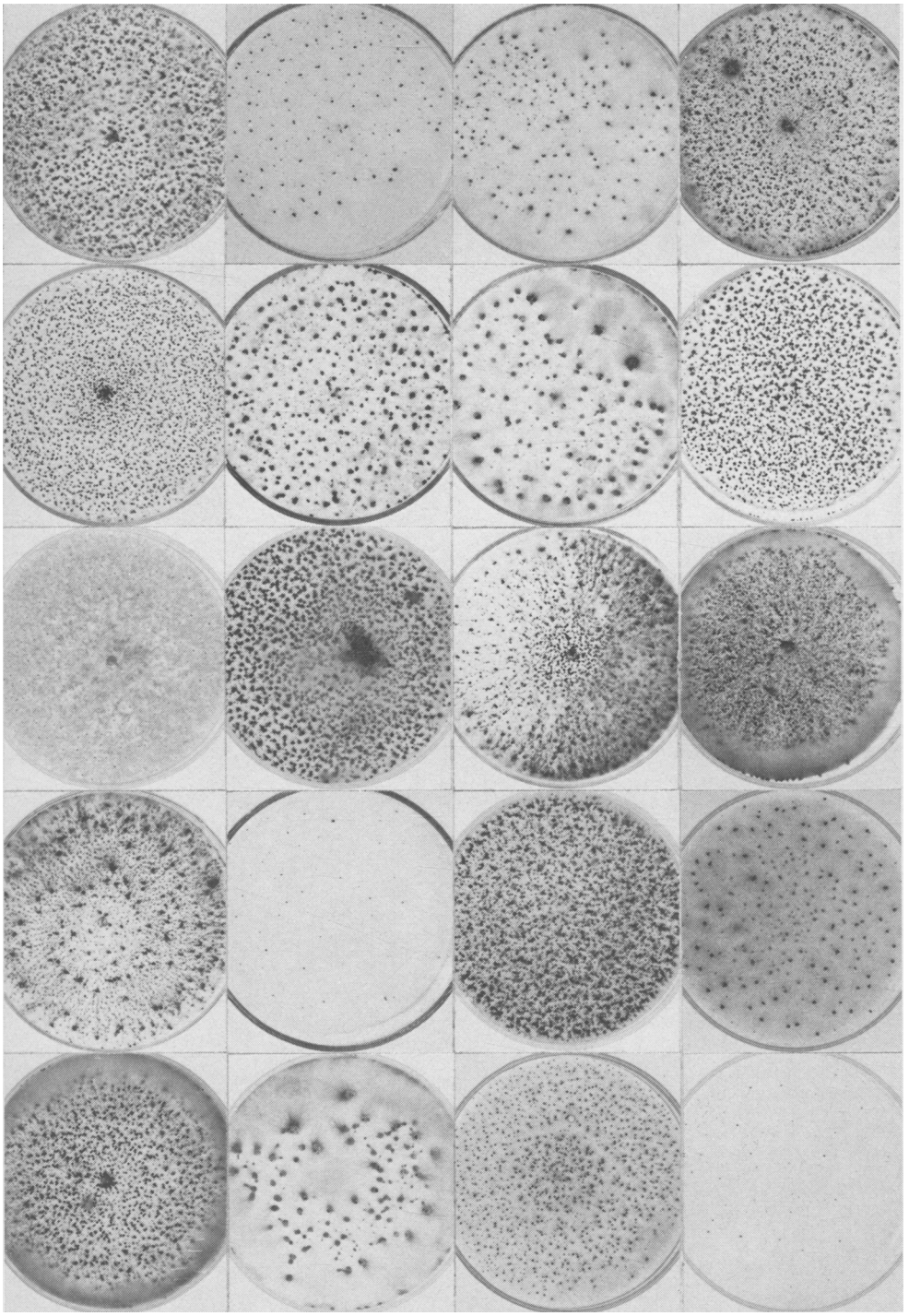

Fig. 3. *Diplodia natalensis* 213, grown for 30 days on basal medium (control) and on various carbon sources. Left to right, top to bottom: glucose, D-ribose, D-galactose, D-fructose, L-sorbose, D-mannose, D-xylose, L-arabinose, lactose, cellobiose, maltose, sucrose, starch, inulin, xylan, cellulose, raffinose, rhamnose, salicin, and basal medium.

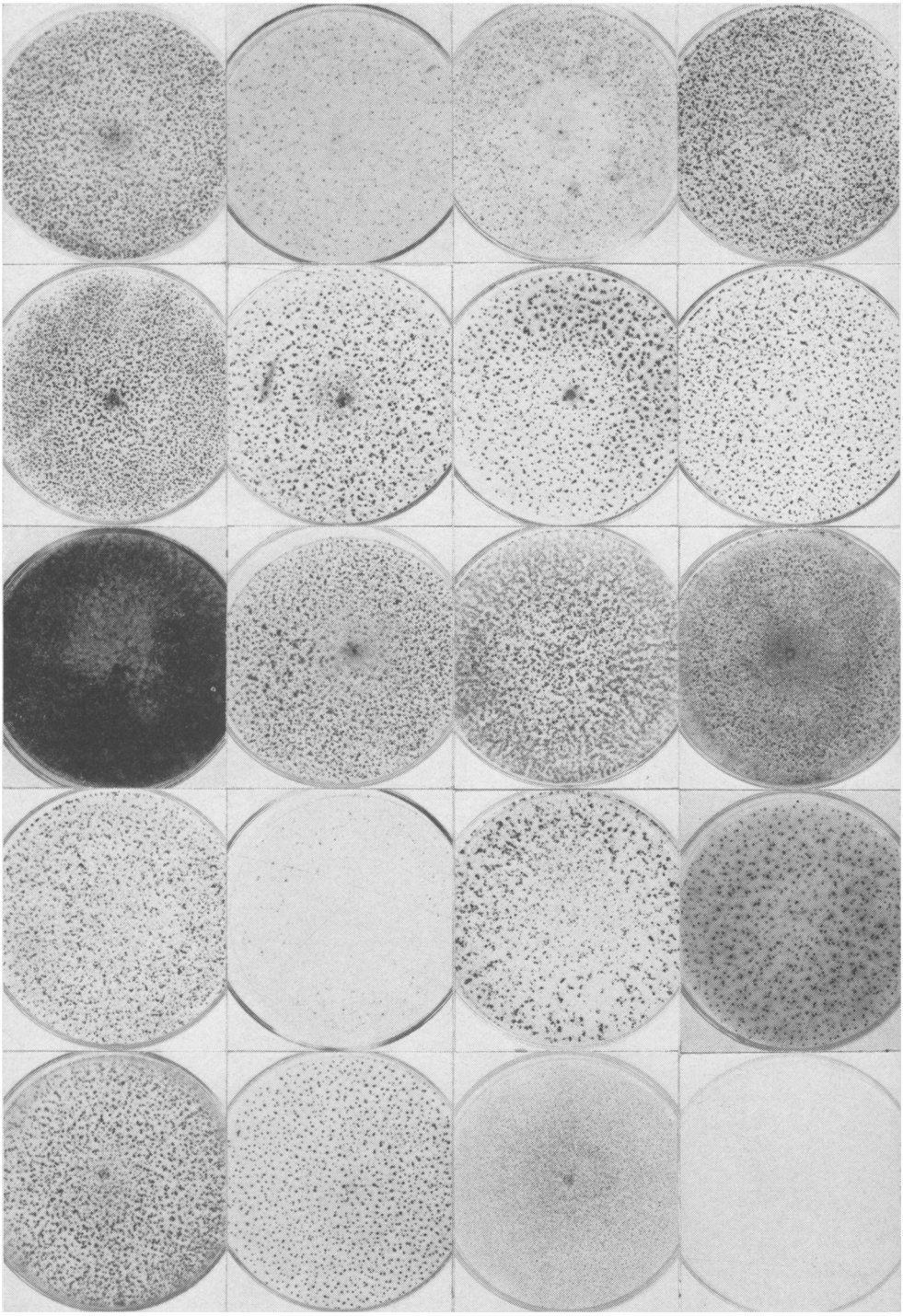

Fig. 4. *Diplodia* sp. 19, grown for 30 days on basal medium (control) and on various carbon sources. Left to right, top to bottom: glucose, D-ribose D-galactose, D-fructose, L-sorbose, D-mannose, D-xylose, L-arabinose, lactose, cellobiose, maltose, sucrose, starch, inulin, xylan, cellulose, raffinose, rhamnose, salicin, and basal medium.

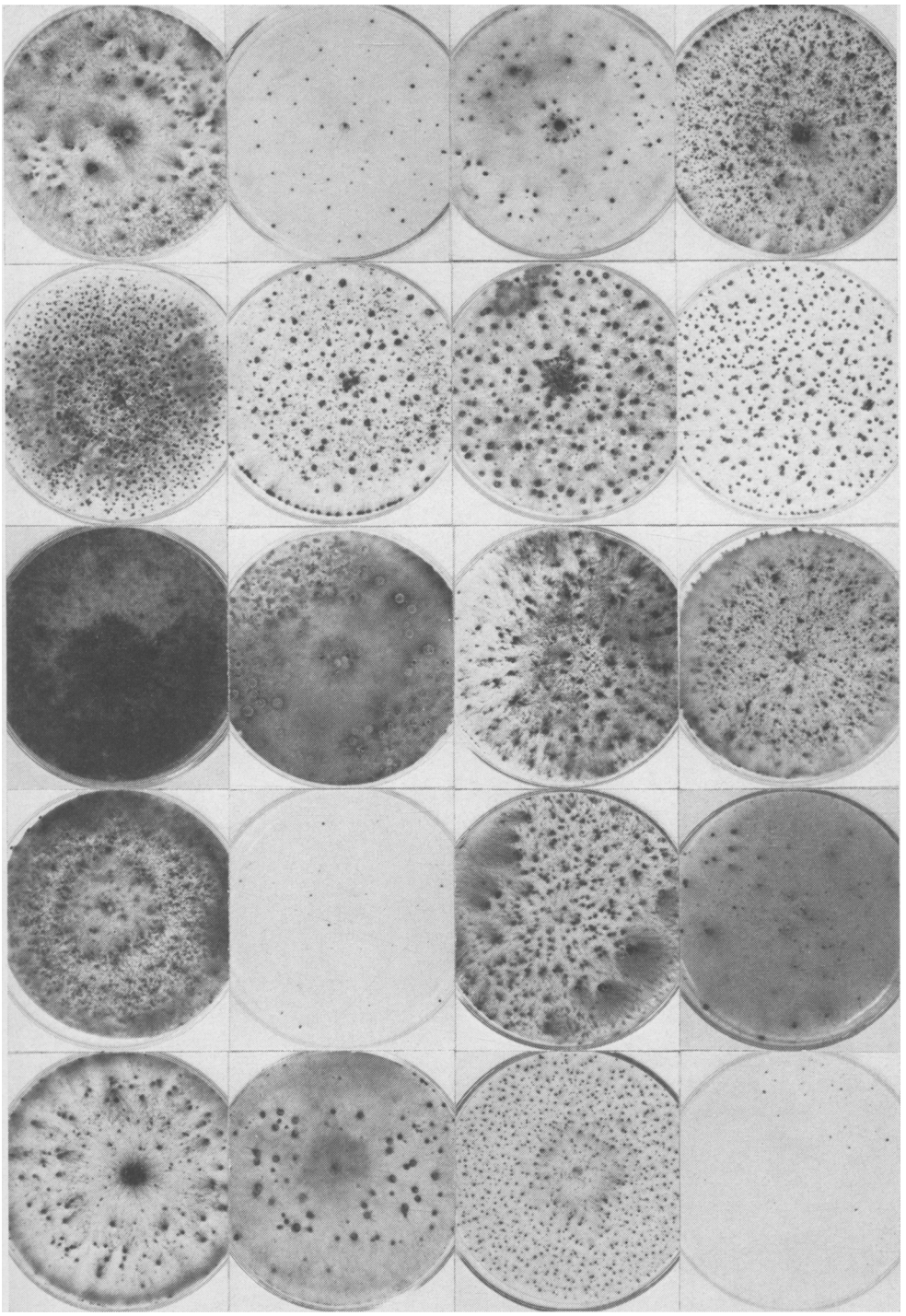

Fig. 5. *Botryodiplodia theobromae* 44, grown for 30 days on basal medium (control) and on various carbon sources. Left to right, top to bottom: glucose, D-ribose, D-galactose, D-fructose, L-sorbose, D-mannose, D-xylose, L-arabinose, lactose, cellobiose, maltose, sucrose, starch, inulin, xylan, cellulose, raffinose, rhamnose, salicin, and basal medium.

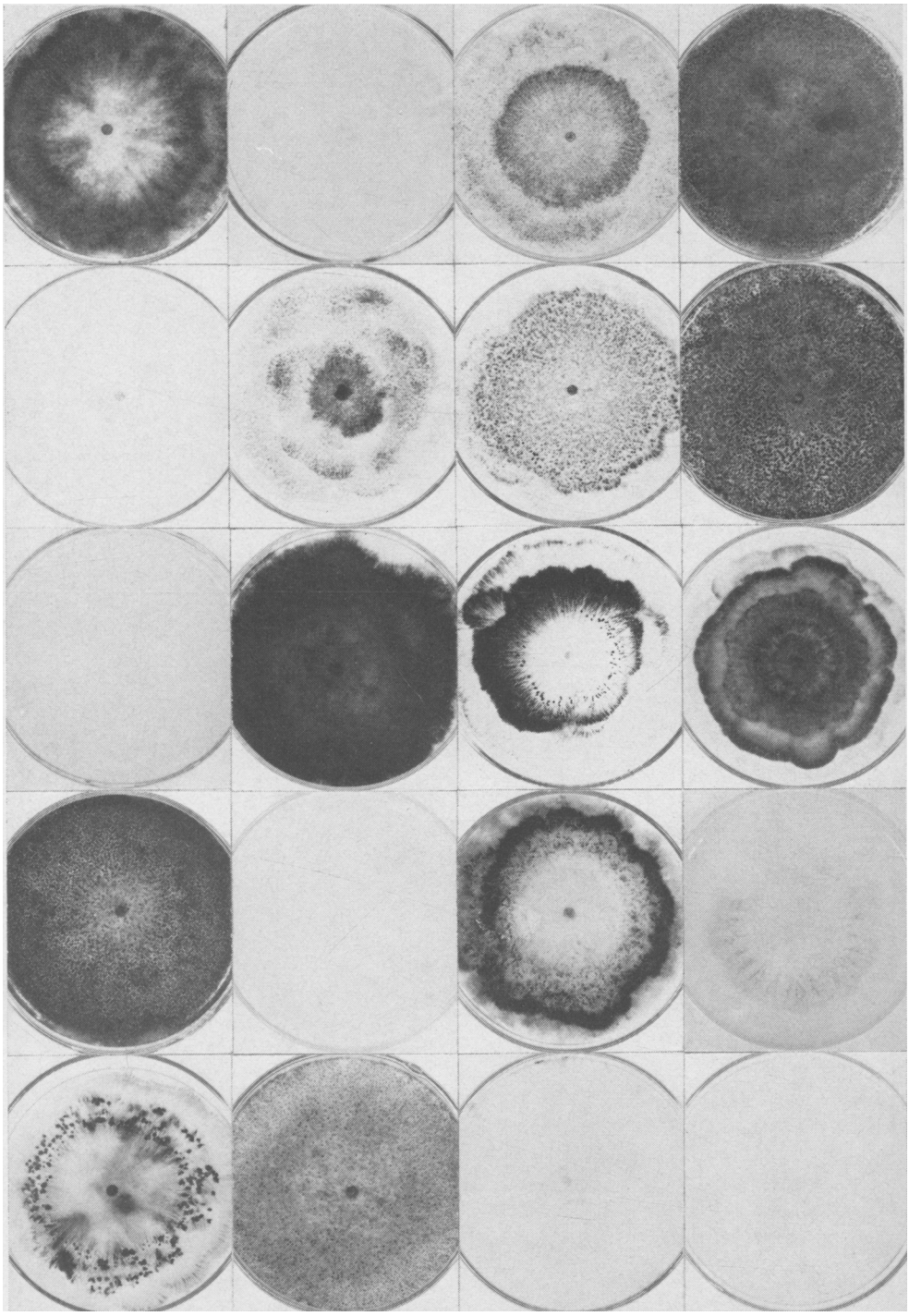

Fig. 6. *Botryodiplodia hypodermia* 29, grown for 30 days on basal medium (control) and on various carbon sources. Left to right, top to bottom: glucose, D-ribose, D-galactose, D-fructose, L-sorbose, D-mannose, D-xylose, L-arabinose, lactose, cellobiose, maltose, sucrose, starch, inulin, xylan, cellulose, raffinose, rhamnose, salicin, and basal medium.

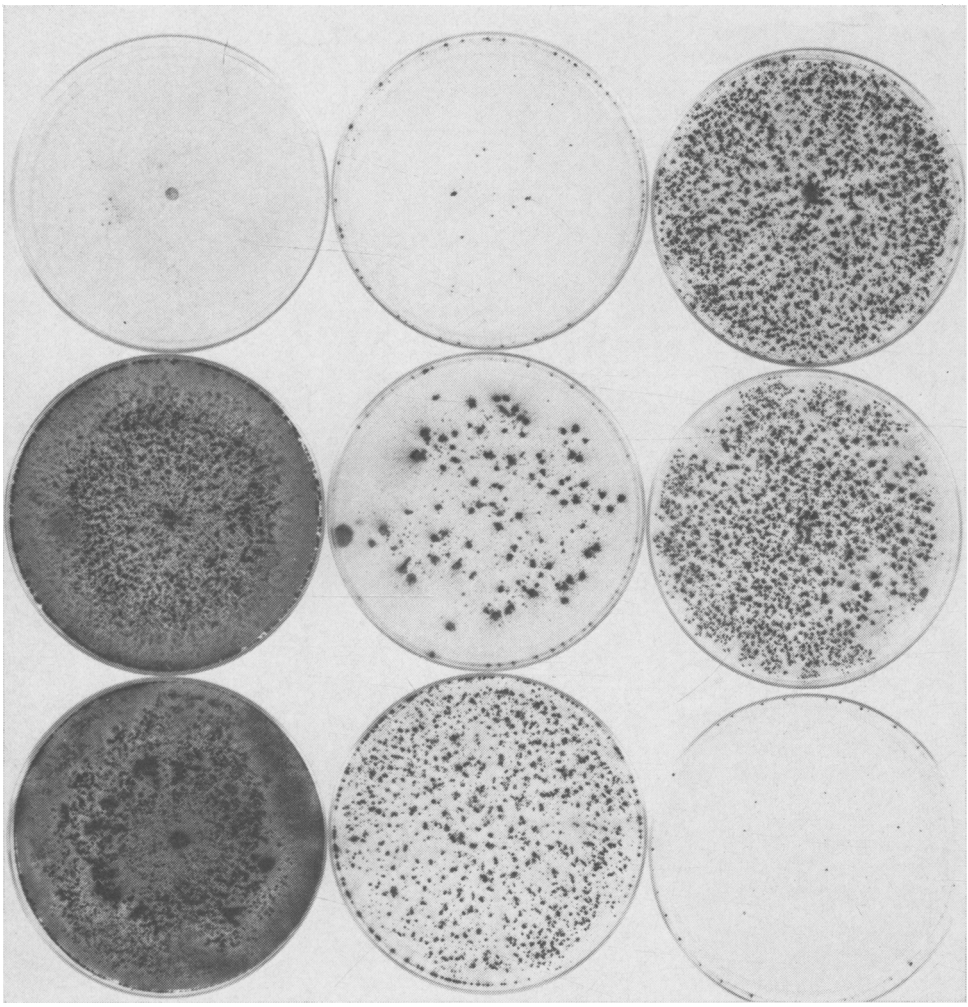

Fig. 8. Growth and sporulation of *Diplodia natalensis* 213 on single and combined carbon sources. Left to right, top to bottom: salicin, inulin, glucose, L-sorbose, salicin + inulin, salicin + L-sorbose, salicin + glucose, inulin + L-sorbose, and inulin + glucose.

mately 275μ and 320μ on salicin and inulin, respectively, and of 717μ when the compounds were combined. Two carbon sources combined sometimes reduced pycnidia size. For example, isolate 213 grown on a mixture of salicin and glucose produced smaller pycnidia than when grown on either of the compounds alone (table 8).

Distribution of pycnidia, i.e., singly or in groups (table 9), varied in some but not all species on some carbon compounds. The same isolates did not vary greatly when grown on either medium A or medium B, plus carbon compound. On medium B (table 9), isolates 157, 213, 130, and 144 produced separate pycnidia on some compounds and separate and grouped pycnidia on others. Isolate 19 produced only separate pycnidia on all carbon sources. In cultures of isolate 29 pycnidia were all separate except on xylose and raffinose, where they were grouped but not in a stroma.

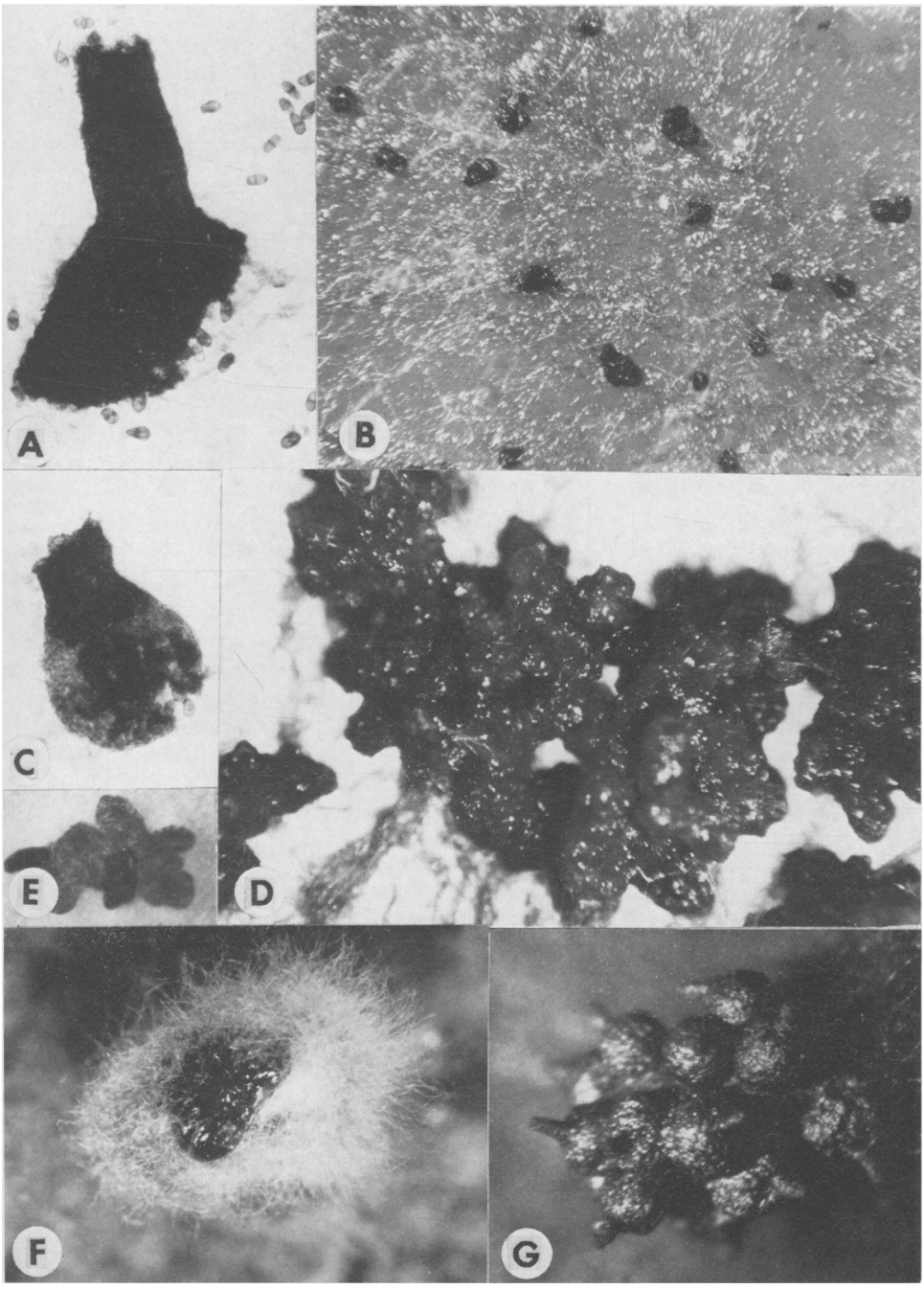

Fig. 9. Fruiting characters of *Diplodia* and *Diplodia*-like fungi: A, pycnidium of *Diplodia natalensis* 147, with long, straight neck, on sorbose; B, single pycnidia of *Botryodiplodia hypodermia* 29 on ribose; C, single pycnidium of *D. natalensis* 147 on inulin (notice thin pycnidial wall); D, aggregates of pycnidia of *B. hypodermia* 29 on raffinose; E, grouped pycnidia of *D. zeae* 130 on arabinose; F, hairy stromata of *B. theobromae* 44 on glucose; G, separate naked pycnidia of *D. natalensis* on potato-dextrose-agar.

TABLE 7

AVERAGE DIAMETERS OF PYCNIDIAL STROMATA OF CERTAIN ISOLATES OF *DIPLODIA* AND RELATED GENERA GROWN ON MEDIUM B SUPPLEMENTED WITH VARIOUS CARBON SOURCES

(Cultures incubated at 24°C for 20 days)

Carbon source	Diameters of pycnidial stromata					
	Species and isolate no.					
	Botryodiplodia hypodermia 29	*B. theobromae* 44	*Botryosphaeria* sp. 19	*Diplodia natalensis* 147	*D. natalensis* 157	*D. natalensis* 213
	μ	μ	μ	μ	μ	μ
Medium B (control)	—	452*	—	—	240	337
L-arabinose	509	1,327	600	550	1,037	960
D-ribose	—	697	373	363	567	590
D-xylose	526	1,553	640	1,030	1,333	1,350
D-fructose	590	1,233	737	493	697	673
D-galactose	323	970	430	887	817	793
D-glucose	380	1,517	517	493	1,353	1,070
D-mannose	243	1,233	813	837	1,043	1,177
L-sorbose	—	873	480	473	477	443
Cellobiose	—	2,203	593	580	927	1,063
Lactose	—	—	—	—	—	—
Maltose	503	1,040	697	537	650	573
Sucrose	—	697	527	500	660	680
Cellulose	—	640	510	477	613	687
Inulin	—	320	250	—	323	290
Starch	397	980	673	580	750	653
Xylan	363	1,003	480	607	783	957
Raffinose	1,030	913	453	433	503	520
Rhamnose	3+0	1,360	463	483	680	1,810
Salicin	—	680	277	263	397	403
D-sorbitol	347	683	—	490	—	527
Linolenic acid	400	517	—	290	—	527
Palmitic acid	—	537	—	407	—	657
Pectin	—	563	—	407	—	—
Sodium acetate	322	400	—	443	—	517
Sodium citrate	—	533	—	—	—	203
Sodium pyruvate	—	720	—	—	—	—
Sodium succinate	287	630	—	403	—	610

* Average of 50 pycnidia.

B. theobromae 44 produced grouped, grouped-fused, and separate pycnidia on glucose, xylose, and rhamnose, and only separate pycnidia on salicin and ribose. *D. natalensis* 213 produced grouped, grouped-fused, and separate pycnidia on fructose, glucose, and xylose, and only separate pycnidia on sorbose and mannose. Isolates of *D. zeae* tended to produce pycnidia in aggregates, with or without stromata, on xylan, cellulose, raffinose, and D-sorbitol.

Orientation of the pycnidia with respect to the substrate was also influenced by the carbon source (table 9). With all isolates, pycnidia appeared either on the surface of the media, or submerged, or both. Pycnidia of isolate 157 appeared only on the surface of 12 of the 23 compounds; were submerged on three; and were both superficial and submerged on the other seven. None were produced on lactose by this isolate.

Orientation of pycnidia of the same species collected from the same location also varied with the carbon source. For example, on fructose, sucrose, and rhamnose, *D. natalensis* 157 produced only superficial pycnidia, whereas iso-

TABLE 8

AVERAGE PYCNIDIAL DIAMETERS OF ISOLATES OF *BOTRYODIPLODIA THEOBROMAE* AND *DIPLODIA NATALENSIS* GROWN ON MEDIUM B SUPPLEMENTED WITH VARIOUS CARBON SOURCES EITHER SINGLY OR IN PAIRS

(Cultures incubated at 24°C for four days)

Taxon and isolate no.	Colony diameters								
	Carbon sources								
	Salicin	Inulin	Sorbose	Glucose	Salicin + inulin	Salicin + sorbose	Salicin + glucose	Inulin + sorbose	Inulin + glucose
	μ	μ	μ	μ	μ	μ	μ	μ	μ
Botryodiplodia theobromae 44	275*	320	917	770	717	503	580	557	343
Diplodia natalensis 157	300	320	593	507	1,007	757	520	630	330
D. natalensis 213	663	387	245	533	593	600	290	537	463

* Average of 50 pycnidia.

late 213 of that species produced submerged or superficial and submerged pycnidia. Location of pycnidia was also affected by light. For example, isolate 213 on fructose, glucose, maltose, or sucrose produced superficial pycnidia under continuous light of approximately 250 ft-c, but produced a mixture of submerged and superficial pycnidia under low light intensity (60 ft-c) and shorter exposure period (8 to 9 hours daily).

Pycnidia of isolate 157 were hairy on control medium A, and naked on control medium B. Pycnidia of different isolates of the same fungus were different. The pycnidia of *Diplodia natalensis* 147 were naked on all carbon sources, whereas those of 157 and 213, all apparently of the same species, were hairy on all carbon sources combined with medium B (table 9). Pycnidia of isolate 213 were naked when grown on basal medium A with added sorbose and also with added sucrose, but had hairs when grown on other carbon sources shown in table 3. On medium B, 213 produced pycnidia with hairs on all but four of the compounds listed in table 9. Observations were not completed on sorbose, maltose, and sucrose. No pycnidia were formed on lactose. Pycnidia of isolates 130, 44, and 19, on all carbon-compound media, had hairs, whereas pycnidia of isolate 29 on the same media did not.

Pycnidiospores. Except for *Sphaeropsis* sp., isolate 218, the isolates are characterized by two-celled spores (Clements, 1909; Bender, 1934; Barnett, 1960). Nevertheless, in this study the spores were usually one- and/or two-celled, depending on the carbon source (fig. 10 A, B). *B. theobromae* 44 produced only one-celled spores on inulin, two-celled spores on glucose, and a mixture of both types on sucrose and rhamnose. The carbon source probably affected the process of spore maturation and septal formation. *D. natalensis* was similarly variable in spore septation. *D. zeae,* in contrast, formed both one- and two-celled spores on all carbon sources tested. Combinations of more than one carbon source also influenced spore septation. For example, isolate 157 produced two-celled spores on glucose, one-celled spores on inulin, and a mixture of both types on a mixture of the two compounds.

Species of *Diplodia* and *Botryodiplodia* are characterized by the production of dark-colored or black spores. In the present study, however, in cultures of

T

PYCNIDIAL CHARACTERS* OF *DIPLODIA* AND *DIPLODIA*-LIKE FUNGI GR
(Cultures exposed to contin

Carbon source	Spec										
	Diplodia natalensis 147				*D. natalensis* 157				*D. natalensis* 213		
	Stromata and pycnidia			Pycnidiospore	Stromata and pycnidia			Pycnidiospore	Stromata and pycnidia		
	Loc.	Status	Hair		Loc.	Status	Hair		Loc.	Status	Hair
Medium B (control)	SM	GN	NH	D	SM	N	NH	D	SM	N	H
Medium B plus:											
L-arabinose	S	GN	NH	D	S	N	H	DW	S	N	H
D-ribose	SM	GN	NH	D	S	N	H	DW	S	N	H
Xylose	S	GN	NH	D	S	N	H	DW	S	GN	H
D-fructose	S	GN	NH	D	S	N	H	DW	SM	GN	H
D-galactose	SM	GN	NH	D	SM	N	H	DW	S	N	H
D-glucose	SM	GN	NH	D	SM	GN	H	D	SM	GN	H
D-mannose	S	GN	NH	D	S	GN	H	DW	S	N	H
L-sorbose	SM	GN	NH	D	SM	N	H	DW	SM	N	—
Cellobiose	S	GN	NH	D	S	GN	H	DW	S	N	H
Lactose	—	—	—	—	O	O	O	O	O	O	O
Maltose	S	GN	NH	D	M	N	H	D	M	N	—
Sucrose	SM	GN	NH	D	S	GN	H	DW	M	N	—
Cellulose	SM	GN	NH	D	S	GN	H	DW	S	N	H
Inulin	SM	GN	NH	D	M	N	H	D	M	N	H
Starch	SM	GN	NH	D	M	N	H	D	S	N	H
Xylan	S	GN	NH	D	S	N	H	D	S	N	H
Raffinose	SM	GN	NH	D	SM	N	H	D	SM	N	H
Rhamnose	SM	GN	NH	D	S	GN	H	DW	M	N	H
Salicin	SM	GN	NH	—	S	N	H	—	S	N	H
D-sorbitol	SM	GN	NH	D	SM	N	H	D	M	N	H
Linolenic acid	S	GN	NH	D	O	O	O	O	S	N	H
Palmitic acid	SM	GN	NH	D	SM	N	H	D	—	—	—
Pectin	SM	GN	NH	D	S	N	H	D	S	N	H

* 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; in a wet matrix; dry and in a wet matrix.
O = did not develop.
— = not observed, or not grown on substrate.
† Pycnidia in groups but no stomata.

the same age, spore color varied from hyaline to black, depending on the isolate and the carbon source (fig. 10 A). For example, *D. zeae* produced hyaline to light-brown spores in all carbon sources tested (fig. 10 C). *D. natalensis* isolates 147, 157, and 213 and *B. theobromae* produced either hyaline spores or a mixture of hyaline and dark-pigmented spores, depending on the carbon source. Spores of isolate 44 were hyaline on inulin, dark-pigmented on sorbose, and a mixture of unpigmented and pigmented on sucrose. Media containing more than one carbon source also had certain effects. With isolate 213, inulin alone or in combination with glucose inhibited spore pigmentation, as did inulin or glucose combined with salicin, and those that were produced were dark brown. Results were the same with isolate 44. Isolate 157, however, produced a mixture of hyaline and brown spores when grown on inulin. Pycnidiospores produced by *Physalospora rhodina* 86 were hyaline except for a few pigmented spores, and those formed by *Sphaeropsis* sp. were dark in color.

Isolates of *D. natalensis* and *B. theobromae* produced striated spores (fig. 10 B), the striation being associated with spore pigmentation and matura-

MEDIUM B SUPPLEMENTED WITH VARIOUS CARBON SOURCES
of 250 ft-c for 30 days)

number

D. zeae 130				*Botryodiplodia theobromae* 44				*B. hypodermia* 29				*Diplodia* sp. 19			
omata and pycnidia			Pycnidiospore	Stromata and pycnidia			Pycnidiospore	Stromata and pycnidia			Pycnidiospore	Stromata and pycnidia			Pycnidiospore
	Status	Hair		Loc.	Status	Hair		Loc.	Status	Hair		Loc.	Status	Hair	
—	—	—	—	SM	N	H	D	SM	N	NH	O	M	N	H	D
N	H	D	S	N	H	W	SM	N	NH	O	M	N	H	D
N	H	W	S	N	H	D	SM	N	NH	O	M	N	—	D
N	H	W	S	GN	H	DW	SM	GN†	NH	O	M	N	H	D
N	H	W	S	N	H	DW	SM	N	NH	O	M	N	H	D
N	H	W	S	N	H	DW	SM	N	NH	O	M	N	H	D
N	H	W	S	GN	H	W	O	O	O	O	M	N	—	D
N	H	W	S	N	H	DW	SM	N	NH	O	M	N	H	D
N	H	W	SM	GN	H	DW	O	O	O	—	M	N	H	D
N	H	W	S	N	H	DW	O	O	O	—	S	N	H	D
N	H	D	O	O	O	O	O	O	O	O	O	O	O	O
N	H	W	S	N	H	DW	SM	N	NH	O	M	N	—	D
N	H	D	SM	N	H	D	SM	N	NH	O	M	N	—	D
N	—	—	S	N	H	W	M	N	NH	O	SM	N	—	D
N	H	DW	M	N	H	D	M	N	NH	D	M	N	H	D
GN	H	DW	S	N	H	D	M	N	NH	O	M	N	—	D
GN	H	W	S	GN	H	D	SM	N	NH	O	M	N	—	D
GN	H	W	S	N	H	D	S	G†	NH	O	M	N	—	D
N	H	W	S	GN	H	W	SM	N	NH	W	M	N	—	D
—	—	—	SM	N	H	O	SM	N	NH	O	SM	N	—	D
N	H	D	S	N	H	D	SM	N	NH	O	SM	N	H	D
O	O	—	SM	GN	—	—	O	O	O	O	O	O	O	O
N	H	D	S	N	H	—	—	—	—	—	—	—	—	—
N	H	W	S	N	H	D	—	—	—	—	—	—	—	—

tion. *D. zeae* 130, *B. hypodermia* 29, and *Sphaeropsis* sp. did not produce striated spores.

Carbon source also affected the size of pycnidiospores (table 10). The following average spore sizes were observed: *D. zeae* 130, $18.3 \times 6.1\mu$ on maltose and approximately $25.3 \times 5.4\mu$ on ribose; *D. natalensis* 157, a range of $23 \times 11.9\mu$, on the control medium, to $30.9 \times 14\mu$ on sorbose. Spore size of isolates 44, 147, and 213 varied less than 3.4μ. Inulin, sorbose, and glucose, alone or in various combinations, had little influence on spore size. There was no spore formation on salicin (table 11).

Release of mature pycnidiospores from the pycnidia, either dry or in wet exudates, varied with carbon source (table 9; fig. 10 D, E). For example, spores of isolate 213 were exuded dry when grown on arabinose, ribose, galactose, sorbose, and cellulose, and in a wet matrix when grown on sodium acetate. Both types were observed on the same plate in the isolate grown on xylose as the sole source of carbon.

Stromata. Two types of stromata were observed: columnar (fig. 11 A, B) and flat (fig. 11 C). The columnar stromata, sometimes branched above the surface of the agar, were approximately 10 mm high (fig. 11 B), and pycnidia were either superficial or embedded in the stromatic tissue. This type of stroma was formed consistently in cultures of *Diplodia* and *Botryodiplodia*. Flat stromata were observed mainly in a culture of *D. zeae* 130.

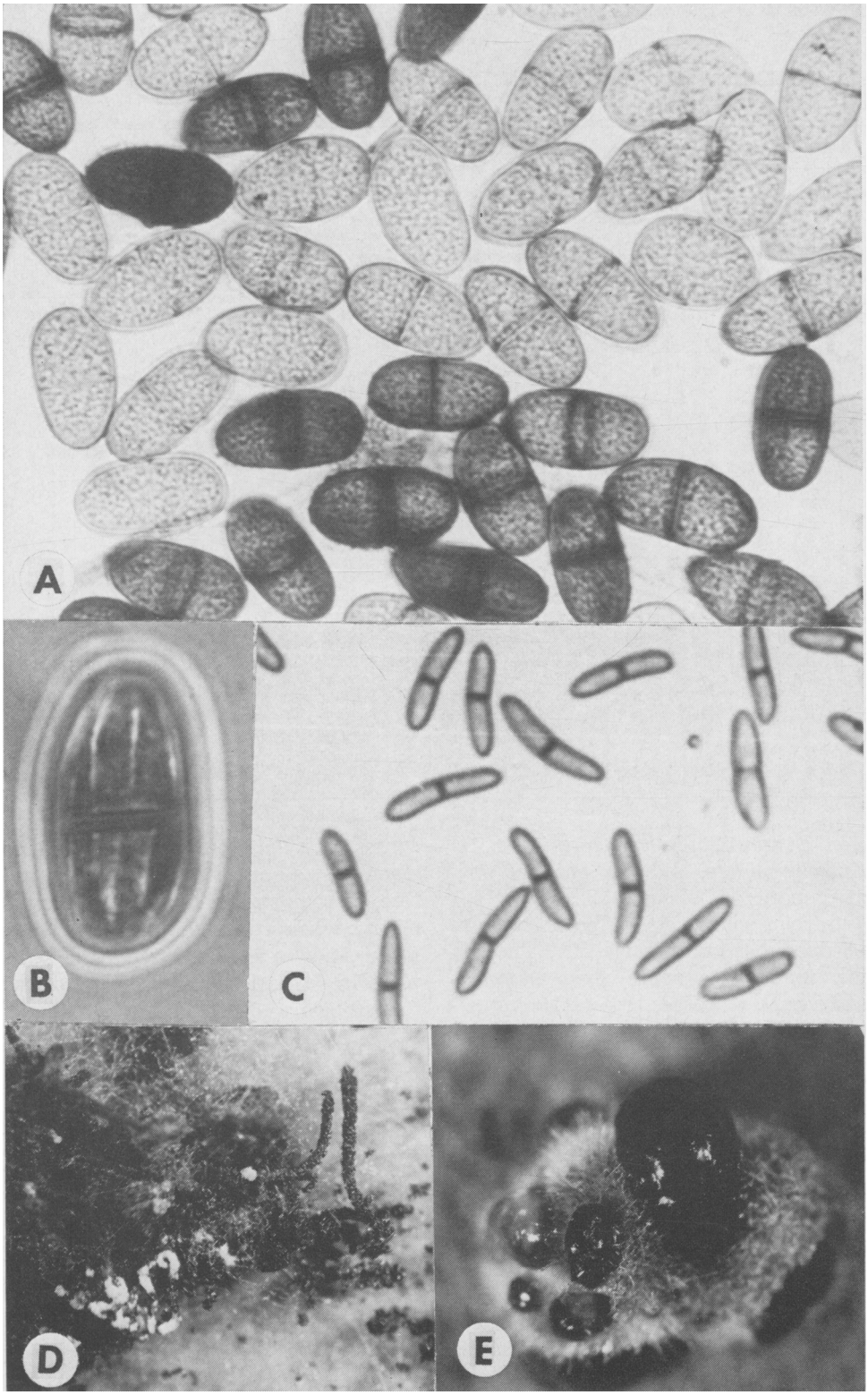

Fig. 10. A, spores of *Diplodia natalensis* 147 on glucose; B, striated spore, typical of those produced by *D. natalensis* and *Botryodiplodia theobromae;* C, spores of D. *zeae* 130; D and E, release of spores from pycnidia in dry and wet matrix, respectively.

TABLE 10

AVERAGE DIMENSIONS OF PYCNIDIOSPORES OF SPECIES OF *DIPLODIA* AND RELATED GENERA GROWN IN MEDIUM B SUPPLEMENTED WITH VARIOUS CARBON SOURCES

(Cultures incubated at 24°C for 30 days)

Carbon source	Species and isolate no.									
	Diplodia theobromae 44		*D. zeae* 130		*D. natalensis* 147		*D. natalensis* 157		*D. natalensis* 213	
	Length	Width	Length	Width	Length	Width	Length	Width	Length	Width
	μ	μ	μ	μ	μ	μ	μ	μ	μ	μ
Medium B (control)	30.4*	14.3	21.1	5.1	22.7	11.9	23.0	11.9	27.2	13.4
Arabinose	27.6	13.4	24.8	6.2	22.9	12.5	28.4	12.7	27.2	14.4
Ribose	28.1	14.0	25.3	5.4	23.5	12.0	27.5	13.4	27.1	12.1
Xylose	28.2	13.9	—	—	23.1	12.0	28.5	12.7	25.9	13.0
Fructose	28.3	14.1	—	—	23.4	12.6	30.5	14.2	26.5	13.9
Galactose	26.5	14.2	22.3	5.5	23.1	12.1	29.3	13.9	25.8	13.5
Glucose	26.1	14.2	19.5	5.8	22.7	12.1	30.2	13.9	26.6	14.6
Mannose	27.2	14.5	17.1	5.3	24.5	12.7	29.1	13.8	27.6	13.9
Sorbose	27.7	14.3	22.2	5.4	23.6	11.7	30.9	14.0	25.3	12.7
Cellobiose	28.5	14.3	19.9	5.7	22.5	11.9	29.3	13.7	26.6	13.3
Lactose	—	—	23.1	5.2	—	—	—	—	—	—
Maltose	26.5	14.1	18.3	6.1	23.4	12.0	26.8	13.3	25.5	12.7
Sucrose	26.9	13.7	22.2	6.1	24.3	13.3	24.9	13.7	24.9	12.8
Cellulose	—	—	22.4	5.2	22.7	11.6	28.4	12.9	27.1	13.1
Inulin	—	—	23.6	5.3	21.6	10.9	27.5	13.1	26.2	13.5
Starch	26.9	14.5	22.9	5.3	22.9	12.9	29.8	13.6	25.5	13.1
Xylan	28.3	14.0	22.7	5.4	22.1	11.4	29.5	13.2	27.2	13.4
Raffinose	28.0	14.8	20.5	5.5	22.9	12.2	28.0	12.9	25.6	13.1
Rhamnose	—	—	19.2	5.5	23.1	11.3	28.7	12.7	26.6	13.2

* Average of 50 pycnidia.

TABLE 11

AVERAGE DIMENSIONS OF PYCNIDIOSPORES OF SPECIES OF *DIPLODIA* AND *BOTRYODIPLODIA*, GROWN IN MEDIUM B SUPPLEMENTED WITH CARBON SOURCES EITHER SINGLY OR IN PAIRS

(Cultures incubated at 24°C for 30 days)

Carbon source	*Diplodia natalensis*				*Botryodiplodia theobromae* 44	
	157		213			
	Length	Width	Length	Width	Length	Width
	μ	μ	μ	μ	μ	μ
Salicin	—	—	—	—	—	—
Inulin	27.8*†	13.1	25.5	13.8	26.7†	13.1
Sorbose	29.1	13.2	25.2	12.9	26.8	13.1
Glucose	28.3	13.8	25.6	13.9	27.5	13.8
Salicin + inulin	28.6	13.8	26.0	13.3	27.9	13.8
Salicin + sorbose	28.1	12.3	27.1	12.9	24.6	13.5
Salicin + glucose	27.2	13.4	24.7	13.0	27.6	13.8
Inulin + sorbose	28.9	13.4	26.0	13.5	26.0	13.3
Inulin + glucose	27.8	13.8	26.1†	13.9	27.2†	12.9

* Average of 50 spores.

† Immature spores, one-celled and nonpigmented.

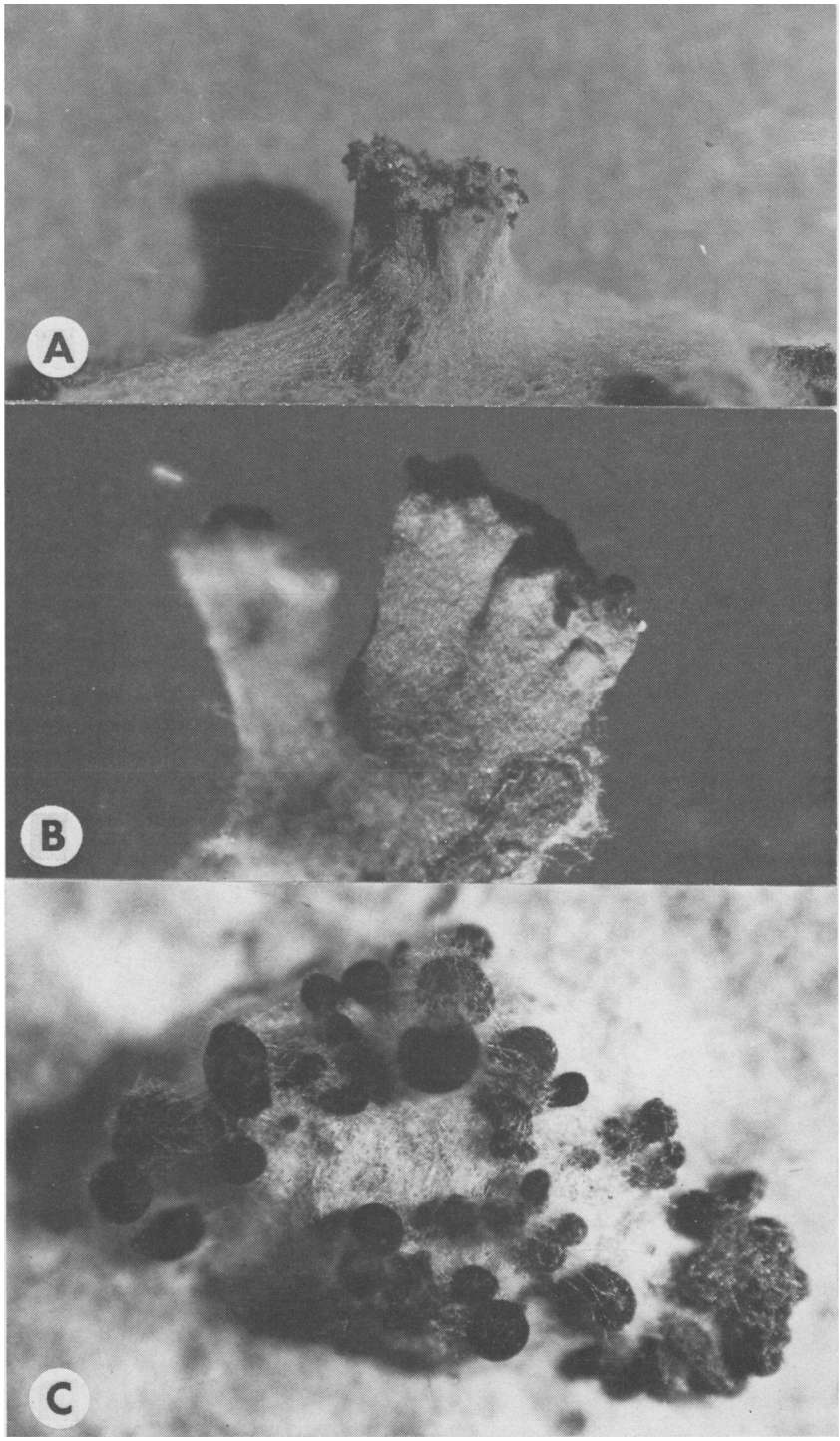

Fig. 11. Morphology of stromata: A and B, simple and branched columnar stromata of *Diplodia natalensis* 213 on glucose; C, flat stromata of *D. zeae* 130 on xylose.

Carbon source influenced the production of stromata. For example, *D. natalensis* 157 formed single, nonstromatic pycnidia on sorbose and ribose, but formed stromata containing pycnidia on glucose, cellobiose, sucrose, and rhamnose. When pycnidia were in groups, stromata were formed. Stromata were produced by *D. natalensis* isolates 157 and 213, but not by isolate 147. The few pycnidia formed by *Physalospora rhodina* were not embedded in stromatic tissue. *B. hypodermia* did not form stromata in spite of aggregation of the pycnidia on certain carbon sources (fig. 9 D). The presence of more than one carbon source in the media influenced the production of stromata. For example, neither salicin nor inulin alone stimulated the formation of stromata, but did so when combined.

DISCUSSION

In general, most of the carbon sources studied were apparently utilized by the various species tested, as indicated by the amount of radial growth on agar. Certain sources were utilized better by some species, however, and some were not utilized at all. Our results agree with those of other workers (Lilly and Barnett, 1953; Brown, 1957; Drake and Moore, 1966) on the utilization of L-sorbose. All species tested utilized this particular sugar very poorly in contrast to glucose, galactose, fructose, etc. Although Lilly and Barnett were able to counteract the effects of sorbose on growth by proper temperature, pH, nitrogen sources, and the like, an isolate of *B. hypodermia* 29 in our study was not able to utilize sorbose even after 35 days of incubation. Species tested were not able to utilize linolenic acid in the first four days, and only a few isolates grew on it during the 30 days of incubation.

Although several workers (Kinsel, 1937; N. E. Stevens and Larch, 1939; Margolin, 1940) reported that *D. macrospora* could not utilize certain monosaccharides, our results indicate that the fungus was able to utilize all carbon sources tested. It may be of significance that all species tested except isolate 107 did grow when cellulose and xylan were the sole sources of carbon, especially since it is known that *D. natalensis* produces a xylanase enzyme (Strobel, 1962, 1963).

The observations and illustrations presented here show that although certain carbon sources did not necessarily stimulate mycelial growth, they supported pycnidia and pycnidiospore formation. This was particularly true with sorbose, which stimulated sporulation but retarded mycelial growth. In contrast, lactose was a good source of carbon for mycelial growth but did not support pycnidial formation (except with *D. zeae* 130). Salicin supported mycelial growth and pycnidial formation, but no pycnidiospores were formed when this compound was the sole source of carbon. In general, pycnidia and pycnidiospore formation was not affected by whether the source was a mono-, di-, or oligosaccharide. Although not necessarily so, the difference may have been due to the specific structure and molecular configuration of the carbon source. For example, ribose, arabinose, and xylose are all pentoses, but ribose supported the least amount of pycnidial formation.

The literature and the present data show that much of the taxonomic confusion about the species included in our study is the result of varying conditions under which fungi have been studied. No extensive studies have been made on the kind and range of variation that

could exist in cultures grown under various conditions. The present evidence indicates that all criteria employed here were unreliable, and varied with the source of carbon in the growth medium. The only character that remained stable enough to be of some value was the size of pycnidiospores. Our results, which are in complete agreement with those of the few workers mentioned earlier, indicate the need for additional studies to establish standard culture conditions for use in taxonomic considerations of these fungi.

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isolate each of *D. zeae, D. macrospora, Botryodiplodia theobromae, Botryosphaeria ribis, Physalospora rhodina,* and a *Sphaeropsis* sp. Isolates were grown in synthetic liquid media and on synthetic agar media, supplemented singly with the different sources of nitrogen. Nitrogen compounds influenced mycelial growth and pigmentation, pycnidial size and orientation with respect to the substrate, presence of hairs on pycnidia, morphology of pycnidia and stromata and pycnidiospores, and exudation of the pycnidiospores. For example, D- and L-valine stimulated vegetative growth, but only L-valine stimulated pycnidial production of most isolates. Lysine and tryptophane retarded the mycelial pigmentation of several isolates, but increased pycnidial production in most species. L-cystine, L-cysteine, D-leucine, and tryptophane inhibited pycnidiospore formation in some isolates of *D. natalensis.*

These data indicate that *Diplodia* and other related genera of fungi may use a wide diversity of nitrogen sources, but that the source of nitrogen may alter the taxonomic characters currently used to delimit this group of fungi.

III. Variation in *Diplodia natalensis* from Grape in California

Single-spore colonies originating from individual pycnidia were compared with each other and with those from different pycnidia from the same grape cane, different canes from the same vineyard, and different vineyards, to evaluate the natural range in variation and stability of taxonomic characters currently used to delimit *Diplodia natalensis* P. Evans. Pycnidia produced in colonies originating from the same sources varied significantly in production of setae, shape, size, loculation, production of paraphyses, and in distribution, i.e., whether single, clumped, or in stromata. Distinct colony types, based mainly on number and distribution of pycnidia and extent of stromata formation, were recognized, and in some cases, as many as four types originated from an individual pycnidium. Colony type *per se* is not considered to be useful for taxonomic purposes. Computer analysis of 70,973 pycnidiospores produced in culture revealed that those from a single pycnidium vary as much in length and width as do those from different collections. Most mature spores produced in culture were dark in color, uniseptate, and characteristically furrowed lengthwise. Biseptate spores were observed occasionally. Spores from cultures had a mean length of $24.77 \pm 2.05\mu$ and width of $12.26 \pm 1.19\mu$, whereas mean length and width of pycnidiospores produced on the canes were $23.25 \pm 2.34\mu$ and $12.03 \pm 1.16\mu$, respectively. Correlation of spore length to width was poor, with $R = .329$.

These results suggest that several genera now recognized in this group are congeneric, and indicate a great need for determination of the inherent variation that these fungi are capable of exhibiting.

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