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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

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

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UNIVERSITY OF CALIFORNIA DIVISION OF AGRICULTURAL SCIENCES

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

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

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III. Variation in *Diplodia natalensis* from Grape in California[1]

ABSTRACT

Single-spore colonies of *Diplodia natalensis* P. Evans 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 this species. Distinct colony types were recognized—in some cases as many as four originating from an individual pycnidium. Colony type *per se* is not considered to be useful for taxonomic purposes. 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.

INTRODUCTION

DIPLODIA CANE BLIGHT is caused by *Diplodia viticola,* which was also reported to be the principal fungus involved in initiating summer bunch rot of grapes (Hewitt *et al.,* 1962). Recently, however, Barbe and Hewitt (1965) reported that the principal fungus involved in summer bunch rot more closely resembled *Diplodia natalensis* P. Evans. Although *D. viticola* has not been adequately described in the literature, it is known to have spores slightly constricted at the septum, from 16 to 22μ long and 7 to 12μ wide, with smooth walls and no paraphyses in the pycnidia (Grove, 1937). According to Evans's (1910) original description, however, mature spores of *D. natalensis* are not constricted at the septum, and measure $24 \times 15\mu$. The species is further characterized by striations or dark bands on the exospore wall parallel to the long axis of the spore, and the pycnidia contain paraphyses that are particularly evident in the young fruit bodies. Many workers since Evans who have dealt with *D. natalensis* have not noted paraphyses for this species. Zambettakis (1953, 1954), however, credited paraphyses to *D. natalensis* and reduced it to synonymy with *Strionemadiplodia frumenti* (El. & Ev.) on the basis of striate spores and paraphyses in the pycnidia.

The literature dealing with these fungi generally presents considerable difficulty in precise identification of genus as well as species. Identification may be complicated further by inadequate knowledge of both the natural variation that may be exhibited by different isolates of a given species and the variation due to the environmental conditions under which the morphological characters are produced and observed. The latter was found to be particularly true by Satour, Webster, and Hewitt (see first and second papers), who reported that the characters used to delimit genera, as well as species, in *Diplodia* and related fungi, showed considerable instability and variation with

[1] Submitted for publication September 10, 1968.

changes in the source of carbon or nitrogen in the media in which the isolates were grown.

This report covers part of an over-all study of experimental taxonomy of *Diplodia* and related fungi. Its specific purpose was to examine a large number of isolates of *Diplodia* from grape in California to determine the amount and range of variation through single-spore analysis and observations of the morphological characters now used to delimit members in this group. In addition, we attempted to determine which of the morphological characters are suitable for use in the taxonomy of the bunch rot fungus. Single-spore colonies of various origins were compared. In addition, morphological characters were observed both on canes selected from the field and in cultures grown in the laboratory, to allow for differences in morphology that may have resulted from cultural conditions.

The analysis is also intended to indicate whether all pycnidia on a single cane are products of the same strain or whether two or more strains may be colonizing the same cane.

MATERIALS AND METHODS

Source and distribution of isolates

Table 1 lists the sources and distribution of the isolates studied. All isolates in the present study were obtained from a five-county area in the San Joaquin Valley where bunch rot is most prevalent. Only isolates from Thompson Seedless (*Vitis vinifera* L.) grapevines were included. Canes that appeared to be infected with *Diplodia* were collected from individual vineyards, wrapped in cheesecloth, and maintained at 3°C until studied. At that time, canes were removed from the cold room, placed on wire-mesh floors in plastic boxes, and incubated at 25°C. Water was added to provide high humidity. When mature fruit bodies appeared, the morphological characters of pycnidia were observed directly on the canes. Single spores isolated from pycnidia produced on the canes were grown to maturity on potato-dextrose-agar (PDA). All PDA was made up as follows: 300 gm peeled potatoes were boiled for 30 minutes, broth was strained through cheesecloth, 15 gm of agar and 20 gm of dextrose were added, plus distilled water to make 1 liter.

Single-spore analysis was carried out as follows: Two canes were selected per collection, and five individual pycnidia on each cane were used as a source of single spores. Thirty single-spore colonies were established from each of the individual pycnidia (fig. 1). The colonies were incubated at 25°C under 24-hour light (Gro-Lux type) of approximately 250 ft-c, and examined within

TABLE 1

SOURCE AND DISTRIBUTION OF *DIPLODIA NATALENSIS* ISOLATES FROM THOMPSON SEEDLESS GRAPE

Collection no.	Location
2	Kern Co.
3A*	Exeter, Tulare Co.
4A	Tulare Co.
6A	Orosi, Tulare Co.
12A	Parlier, Fresno Co.
17A	Madera Co.
21A	Exeter, Tulare Co.
24A	Bakersfield, Kern Co.
26A	Biola, Fresno Co.
27A	Biola, Fresno Co.
30A	Livingston, Merced Co.
34A	Fowler City, Fresno Co.
157	Fresno Co.
164	Fresno Co.
181, 191, 194, 195	Tulare Co.
186, 199, 200, 201	Fresno Co.
187, 175, 202, 203	Fresno Co.
189, 204, 207, 208	Fresno Co.
190, 9, 12, 17	Fresno Co.

* Collections designated with "A" are considered regions in the nested analysis.

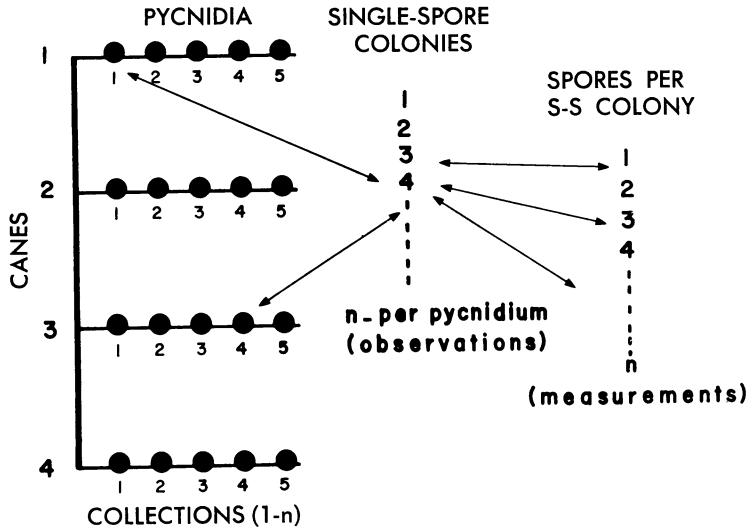

Fig. 1. Diagram of procedure for single-spore analysis of *Diplodia natalensis*. Five pycnidia from each of four Thompson Seedless canes per collection were used as a source of single spores. Twenty spores from each of 15 single-spore colonies obtained from each pycnidium were measured.

26 to 32 days after isolation of the spores.

Observations on pycnidial morphology, presence or absence of paraphyses, and spore characters were made under the microscope at the time the spore samples were taken from the single-spore colonies. All single-spore colonies were photographed for comparison both at colony maturity and later. Thus a large number of colonies could be compared at completion of the single-spore analysis.

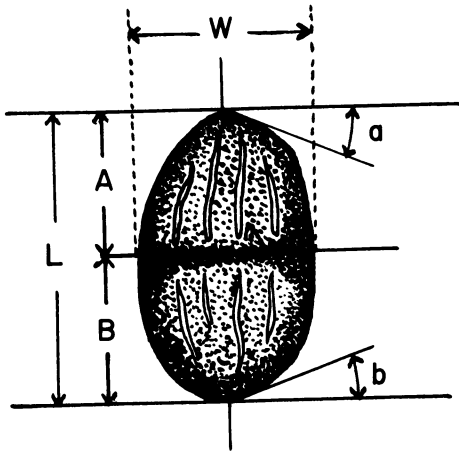

Fig. 2. Diagram of typical pycnidiospore of *Diplodia natalensis*. Labeling indicates variables measured. See table 8 for data.

Individual single-spore colonies produced in culture and from pycnidia obtained from the canes were measured as follows: Spores were mounted on microscope slides in a 10 per cent glycerine solution, and photographed on 35-mm film with the Zeiss Ultraphot. A stage micrometer was also photographed at the same time and the same magnification. Measurements were obtained by projecting the micrometer, obtaining a standard measure, and then measuring spores as projected.

The spores were photographed, and spore shape and morphology were studied further by measuring the distances from the central septum to either end of the spores. Also measured was the angle of curvature at the terminal ends of the spores (fig. 2). Statistical analyses of the data were made with an IBM 7040 computer.

RESULTS AND OBSERVATIONS

Colony types

To evaluate natural range in variation and stability of colony type, single-spore colonies originating from individual pycnidia were compared with each other and with those of different pycnidia from the same grape cane, from different canes in a given vineyard, and from vineyards in different locations. As seen in figure 3, a large range of colony types was observed from the 12 regions analyzed. Colonies differed in amounts of aerial mycelium, distribution, production, and numbers of stromata, and presence or absence of individual pycnidia without stromata. Some colonies contained very few pycnidial initials or stromata, whereas the surface of others was nearly covered either with stromata alone, individual pycnidia, or a combination of both. Single-spore colonies from a single region either were all nearly identical or, in some cases, involved several colony types. This variation applied not only to regions but also to canes and pycnidia within regions. For instance, in Region 1 all single-spore colonies obtained from two canes and 10 pycnidia were virtually identical (see fig. 4). A single cane from Region 12A, in contrast, yielded nearly as much variation in colony types as was found in the entire collection (fig. 5). Individual pycnidia generally yielded only one colony type, although sometimes pycnidia from a single cane or from two canes from a single region yielded varying colony types. Usually, not more than two colony types were observed from an individual pycnidium, but in a few cases six distinct types from an individual cane were noted. Since a distinct colony type may characterize a particular strain, this observation suggested that a single cane may be infected by two or more strains. The range in variability observed indicates that colony type *per se* is not suitable as a taxonomic character. This is particularly true in view of the fact that colony type is for the most part based on the production of stromata and the distribution of pycnidia. On this basis, no attempt is made to characterize a particular colony type even though, as stated, single-spore colonies may often be divided into a number of discrete groups, that is, 1-n distinct colony types.

Stromata and pycnidia

The characters of the pycnidium at present considered to be of taxonomic value in identifying members of this group of fungi include: whether or not the pycnidium is hirsute or glabrous; superficial or submerged; produced in, on, or without a stroma, or beaked; the loculation or the complexity of the spore chambers; and the presence or absence of paraphyses. All these characters were observed, to determine the stability, range, and variation in the collection under study. The observations involved a total of 2,806 single-spore colonies from 132 individual pycnidia. Of these, all contained multiloculate stromata, and a few single pycnidia were produced without a stroma. Paraphyses were present in all cases, at least in younger developing pycnidia. These structures often became very difficult to find in the older fruit bodies. All of the colonies contained pycnidia that were either hirsute or associated with some type of mycelial outgrowth (fig. 6). Some colonies contained naked pycnidia produced on the surface of fuzzy stromata (fig. 6), and in some cases both were observed in the same colony and often on the same stromatic mass.

Species are also determined in *Di-*

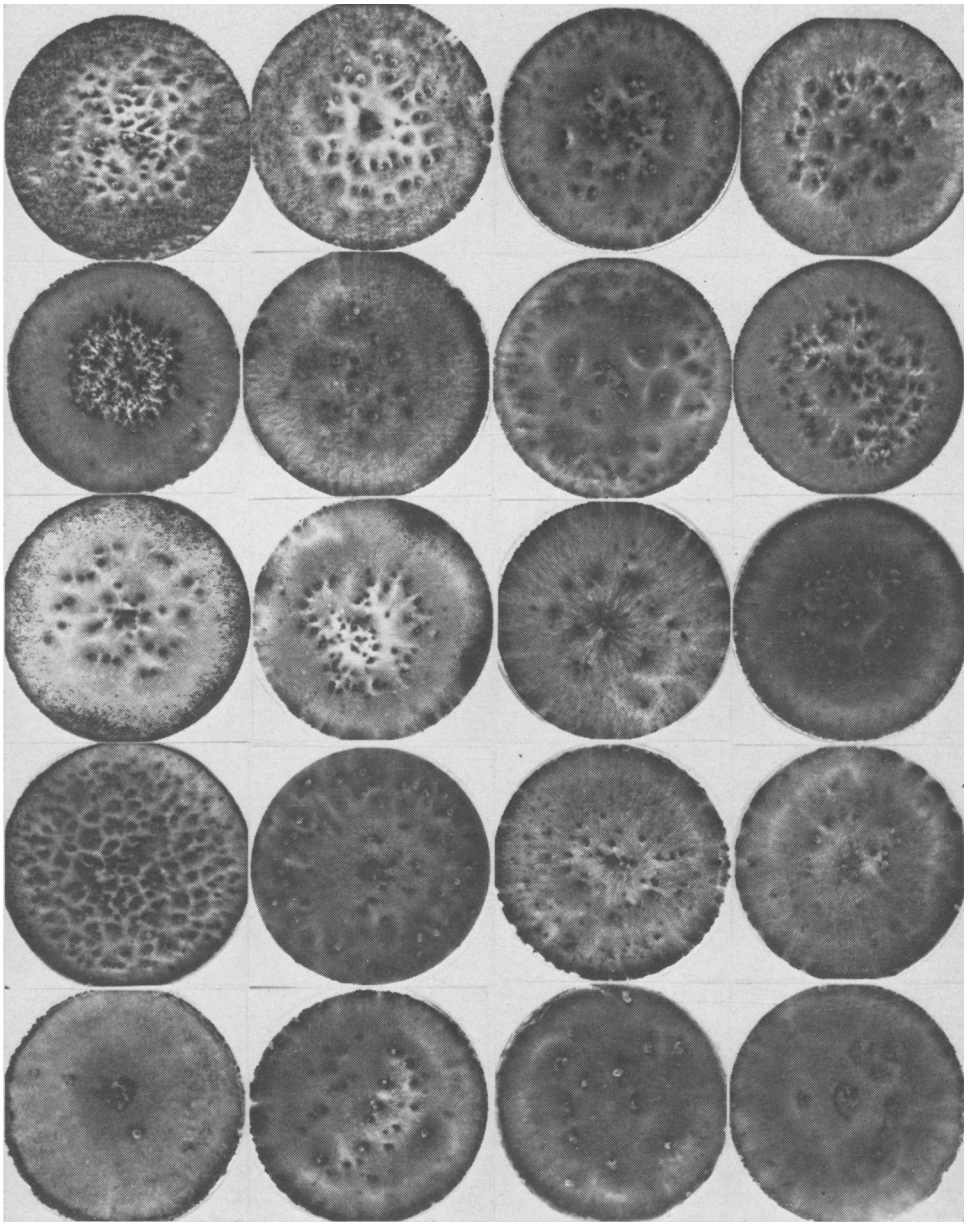

Fig. 3. Colony types obtained in single-spore analysis of *Diplodia natalensis.*

plodia by whether spores are produced dry or in a wet, slimy matrix. All colonies produced spores in a wet matrix at some time during their development; and production in dry cirri was also observed (fig. 6). In the majority of colonies the spores, in most cases, were produced both dry and in a wet matrix.

Pycnidia diameters were extremely variable. For example, the mean diameter for pycnidia produced on grape canes from Region 34 was 225.3μ

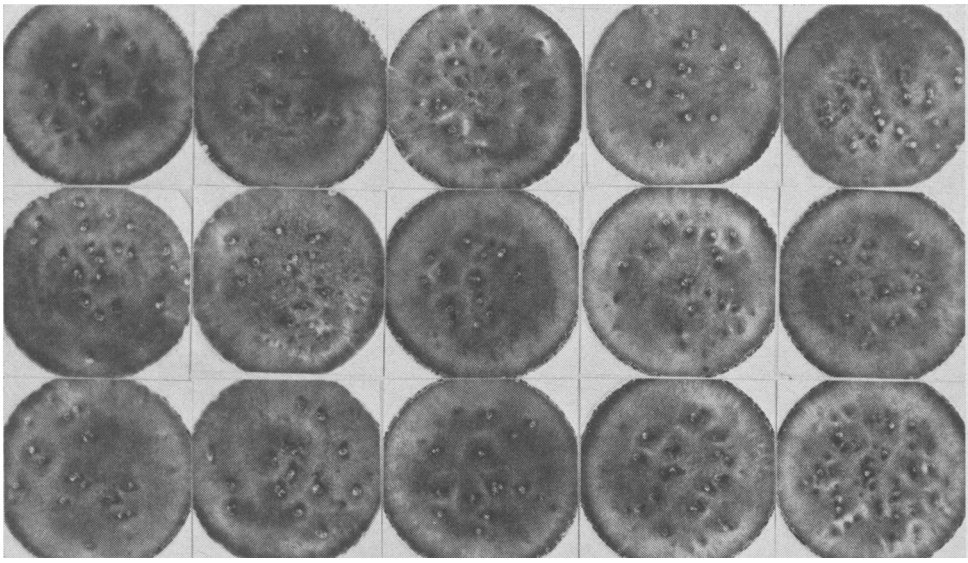

Fig. 4. Colony types obtained in single-spore isolations of *Diplodia natalensis* from a single Thompson Seedless grape cane. Note uniformity in this sample.

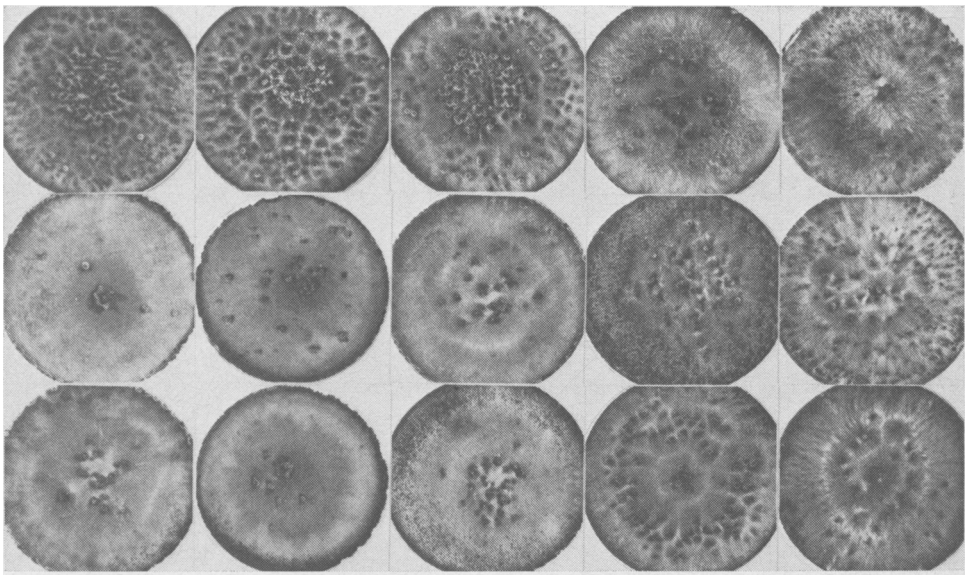

Fig. 5. *Diplodia natalensis* colony types obtained from a single Thompson Seedless grape cane. Note distinct types. (Cf. fig. 4.)

(range, 625 to 93), compared with 362μ from Region 4 (range, 752 to 156). Measurement of pycnidial diameters in cultures was extremely difficult and was soon abandoned because of variation in the amounts and types of stromata in or on which the pycnidia were produced (fig. 6). All colonies contained stromata that were multiloculate in that each had its own opening through which spores were liberated. Table 2 gives means and ranges of pycnidial diam-

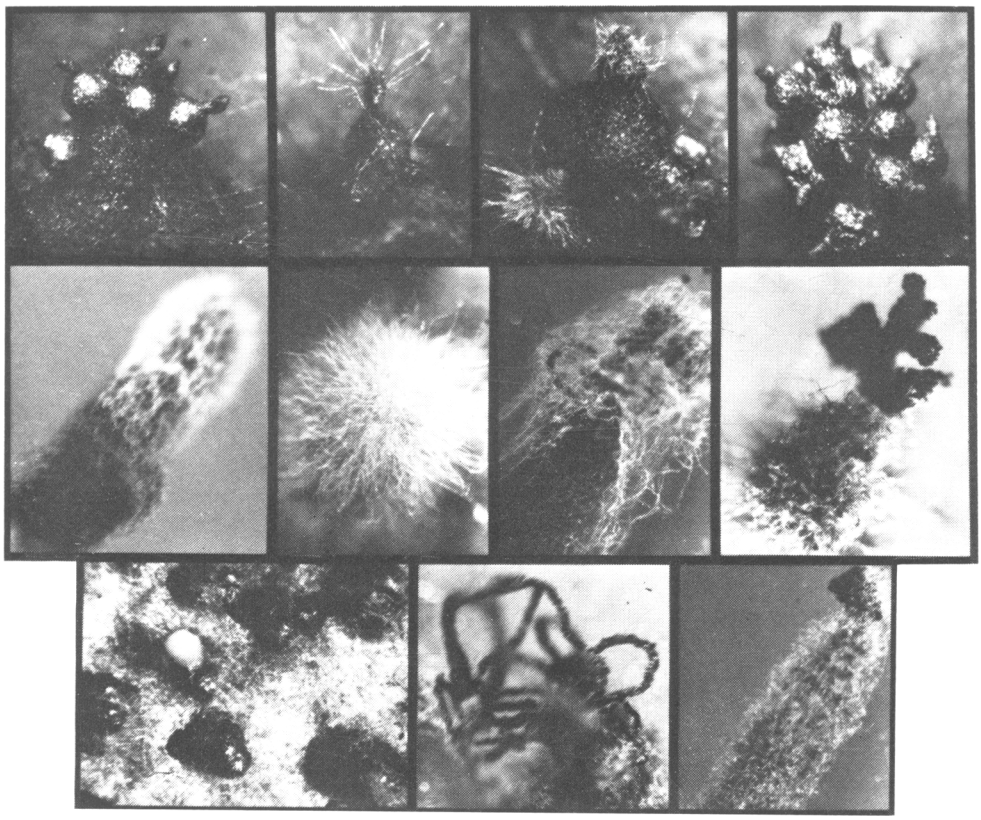

Fig. 6. Variation in pycnidial characters. (All figures from single-spore analysis of *Diplodia natalensis.*)

eters as observed on the canes from the 10 regions. Analysis of variance showed that these values differ significantly between collections, with an F value of 26.3, compared with an expected 1.89 at the .05 level.

Pycnidiospores

Zambettakis (1953, 1954) considered one of the more important and useful taxonomic characters in this group to be ornamentations of the exospore wall. On the basis of furrowed ridges (striations) running lengthwise on the spore of *Diplodia natalensis*, coupled with the production of paraphyses, Zambettakis placed this fungus in the genus *Strionemadiplodia* and considered it to be a synonym of *S. frumenti.* The pycnidiospores observed in the cultures isolated from grape in California exhibited considerable uniformity in that spores were hyaline when young, with a thick wall, becoming dark and septate at maturity. Mature spores in all of the isolates observed were characteristically furrowed as described by Evans (1910) and as figured by Barbe and Hewitt (1965). On rare occasions biseptate spores were observed.

The lengths and widths of single pycnidiospores originating from single-spore cultures isolated from individual collections were compared with those of spores from pycnidia produced on the grape canes. The results, given in table 3, show that spores produced in culture differ little in width-length value from

spores produced on grape canes from the field. Since pycnidiospore size and morphology appeared to be the most stable characters studied, additional analyses were carried out to determine the variation in spore size among the individual collections, the canes included in each collection, the pycnidia on individual canes, and the single-spore colonies originating from the pycnidia. Since spores produced in culture differed little from spores on natural substrates, values were obtained only from colonies grown in culture. The following analyses refer to the 10 original collections. To give a balanced set of data, two canes were chosen per collection, with three pycnidia per cane. Table 4 shows that in all cases analyses of variance in both width and length gave significant F values for the collections, canes, pycnidia, or individual colonies. To determine the source of variations (i.e., between canes within a collection, pycnidia from a single cane, or between single-spore colonies originating from a single pycnidium), a nested type of analysis was used. Table 5 shows the results of comparisons between a balanced set of data obtained from 10 (regions) collections with two canes per collection, three pycnidia per cane, 15 single-spore colonies per pycnidium, and 25 spores per colony. In terms of width and length of spores, differences between single-spore colonies originating from the

TABLE 2

MEAN DIAMETER AND RANGE IN SIZE OF *DIPLODIA NATALENSIS* PYCNIDIA PRODUCED ON CANES OF THOMPSON SEEDLESS GRAPE FROM 10 REGIONS IN CALIFORNIA*

Region no.	Mean	Range
	μ	μ
3A	303.1†	537–197
4A	362.1	752–156
6A	323.4	687–125
12A	323.0	625–156
17A	214.4	500–93
21A	211.7	375–125
24A	322.6	625–197
27A	389.8	625–250
30A	361.7	562–197
34A	225.3	625–93

* Analysis of variance among regions, $F = 26.33$. Expected F values = 1.89 (.05) and 2.43 (.01).
† Represents 60 pycnidia per collection.

TABLE 3

AVERAGE LENGTH AND WIDTH OF *DIPLODIA NATALENSIS* PYCNIDIOSPORES PRODUCED IN POTATO-DEXTROSE-AGAR CULTURE AND ON THOMPSON SEEDLESS GRAPE CANES FROM THE FIELD

Region	Spores in culture		Spores on field canes	
	Width	Length	Width	Length
	μ	μ	μ	μ
30A	11.91 ± 1.19*	24.28 ± 1.99	11.93 ± 1.66†	22.80 ± 2.39
34A	11.62 ± 1.12	24.21 ± 2.07	11.60 ± 1.09	22.55 ± 1.88
12A	12.33 ± 1.30	24.80 ± 2.22	11.51 ± 0.85	23.27 ± 3.26
17A	12.55 ± 1.40	25.63 ± 2.32	12.02 ± 1.34	22.91 ± 2.69
21A	12.30 ± 1.40	25.44 ± 2.50	13.40 ± 1.43	24.58 ± 2.48
3A	11.41 ± 1.25	23.99 ± 2.13	11.80 ± 1.08	23.57 ± 2.05
27A	11.82 ± 1.23	23.84 ± 2.09	12.70 ± 1.39	24.26 ± 2.29
4A	11.70 ± 1.23	23.58 ± 2.10	11.65 ± 0.95	22.55 ± 2.00
6A	13.47 ± 0.94	25.86 ± 1.54	11.98 ± 0.84	23.15 ± 2.02
24A	13.29 ± 0.91	25.95 ± 1.61	11.73 ± 1.00	22.88 ± 2.50
Grand means:	12.26 ± 1.19	24.77 ± 2.05	12.03 ± 1.16	23.25 ± 2.34

* Each value represents observations of 2,250 spores per region.
† Each value represents observations of 80 spores per region.

TABLE 4

F VALUES OBTAINED IN ANALYSIS OF VARIANCE OF PYCNIDIOSPORE WIDTH AND LENGTH WITHIN INDIVIDUAL COLLECTIONS OF *DIPLODIA NATALENSIS* FROM THOMPSON SEEDLESS GRAPE

Region	*F* values from comparisons between:					
	Canes		Pycnidia		Colonies	
	Width	Length	Width	Length	Width	Length
30A	19.38	0.15	1.85	6.60	6.62	3.75
34A	0.002	3.58	427.20	0.27	9.57	5.57
12A	2.59	22.86	0.38	0.04	19.29	6.52
17A	1.53	1.24	0.67	0.80	18.48	7.19
21A	3.79	69.84	0.26	0.01	12.09	4.31
3A	0.61	0.32	1.61	3.08	10.47	6.95
27A	0.21	1.78	4.57	0.56	13.55	5.51
4A	8.02	5.62	0.12	0.17	9.32	4.69
6A	2.01	1.58	0.49	0.63	6.63	1.58
24A	9.87	152.96	0.10	0.006	5.22	3.03
Required *F* values:						
.05	1.83	1.83	1.40	1.40	1.11	1.11
.01	2.32	2.32	1.59	1.59	1.15	1.15

same pycnidium were as great as or greater than the differences between colonies originating from pycnidia on the same cane or on different canes or even from different collections. Furthermore, within a given collection the differences between pycnidia and pycnidiospores produced on a single cane were not always significantly different from the differences observed between those produced on companion canes from the same collection or even from different collections. As indicated in the table, however, when differences between single-spore colonies from individual pycnidia were analyzed, in all but three cases the *F* values obtained showed a significant difference at the .01 level. Thus, the significant differences detected in spore size indicate that this character is as variable in colonies originating from a single pycnidium as it is even between spores obtained from different collections.

To determine the correlation between width and length of these spores, a regression analysis between width and length was carried out. The results indicated that width and length are not well correlated, since the correlation coefficient, R, was equal to .329. Further tests indicated, however, that width and length are not completely uncorrelated. Even though statistically significant differences were detected between spores from single-spore colonies from individual pycnidia, the actual dimensions of the spores, as shown in table 3, indicate that spore width and length are in all probability the most stable characters of the isolates studied, and the differences detected are an expression of the inherent variability of spore size. To check this assumption further, an additional 22 separate, individual isolates were obtained from Thompson Seedless grape, and 25 single-spore colonies were established from each. Table 6 gives the mean width, length, and *F* values obtained in an analysis of variance comparing the individual colonies of each isolate on a basis of spore width and length. As the table shows, the values correlate rather well with those obtained for the 10 original collections. Likewise, in this analysis, variation between spores produced in single-spore colonies from individual isolates ap-

TABLE 5

F VALUES OBTAINED FROM ANALYSIS OF VARIANCE, WHERE *DIPLODIA NATALENSIS* COLONIES ARE NESTED WITHIN PYCNIDIA, PYCNIDIA WITHIN CANES, AND CANES WITHIN REGIONS, SHOWING SOURCES OF VARIATION OF SPORE WIDTH AND LENGTH WITHIN INDIVIDUAL COLLECTIONS

(Observations include measurements of 25 spores from each of 15 single-spore colonies isolated from each individual pycnidium, with a total of 60 pycnidia analyzed.)

Region	Between pycnidia		Between colonies			Region	Between pycnidia		Between colonies		
	W	L	Pyc.	W	L		W	L	Pyc.	W	L
30A:						3A:					
Cane 1..	0.073	3.07	1	5.308	6.00	Cane 1..	17.58**	20.58**	1	18.62	30.10
			2	10.62	4.00				2	9.37	5.03
			3	5.28	3.61				3	15.06	3.29
Cane 2..	3.328*	3.59*	1	2.85	3.98	Cane 2..	1.24	8.98**	1	5.68	2.61
			2	5.94	1.62				2	5.37	3.96
			3	10.99	3.22				3	6.12	1.34
34A:						27A:					
Cane 1..	2.90	0.34	1	12.92	6.85	Cane 1..	29.53**	5.58**	1	6.04	7.75
			2	8.08	5.62				2	2.07*	1.87*
			3	15.25	6.44				3	7.55	2.92
Cane 2..	2.82	1.12	1	10.08	8.02	Cane 2..	2.57	0.87	1	17.59	8.43
			2	5.49	3.60				2	16.98	5.59
			3	5.81	3.32				3	27.46	6.10
12A:						4A:					
Cane 1..	5.60**	2.45	1	8.00	2.41	Cane 1..	0.93	1.47	1	19.67	7.99
			2	10.32	5.50				2	14.89	7.39
			3	13.45	3.81				3	7.81	4.52
Cane 2..	0.88	0.08	1	18.99	7.32	Cane 2..	4.57*		1	6.65	2.52
			2	29.56	8.44				2	4.87	2.44
			3	36.86	9.81				3	5.66	3.11
17A:						6A:					
Cane 1..	12.88**	18.46**	1	11.95	4.59	Cane 1..	11.12**	0.57	1	2.56	2.25
			2	10.15	6.74				2	3.64	1.13
			3	11.70	5.02				3	5.51	2.54
Cane 2..	0.02	0.25	1	11.50	8.14	Cane 2..	2.07	1.61	1	12.82	3.89
			2	35.78	9.06				2	6.23	2.13
			3	26.97	8.61				3	7.38	3.15
21A:						24A:					
Cane 1..	15.97**	4.98*	1	23.07	8.02	Cane 1..	3.41*	0.20	1	4.29	2.81
			2	9.75	2.87				2	8.43	4.75
			3	8.44	3.28				3	2.57	3.64
Cane 2..	4.12*	1.21	1	5.15	3.88	Cane 2..	1.33	1.15	1	3.88	2.10*
			2	7.79	5.51				2	4.67	2.56
			3	15.69	3.47				3	7.72	2.39
Required *F* values:											
.05.....	3.22	3.22		1.72	1.72		3.22	3.22		1.72	1.72
.01.....	5.15	5.15		2.12	2.12		5.15	5.15		2.12	2.12

TABLE 6

ANALYSIS AND MEAN WIDTH AND LENGTH OF PYCNIDIOSPORES PRODUCED IN SINGLE-SPORE CULTURES FROM 22 ISOLATES OF *DIPLODIA NATALENSIS* FROM THOMPSON SEEDLESS GRAPE

Isolate	Mean width	Observed *F* values between colonies†	Mean length	Observed *F* values between colonies†
	μ		μ	
9	11.61 ± 0.89	2.06*	24.52 ± 2.05	2.60**
189	11.90 ± 1.14	1.90	25.06 ± 2.01	2.41*
186	11.90 ± 0.91	2.09*	25.54 ± 1.97	2.99**
187	11.81 ± 0.89	2.04*	25.39 ± 2.14	2.42
181	11.68 ± 1.14	2.54**	25.44 ± 2.12	1.81
175	10.76 ± 3.72	367.62**	21.68 ± 7.45	4.54**
164	10.14 ± 3.49	511.99**	21.10 ± 7.32	3.47**
157	13.22 ± 1.20	2.99**	28.38 ± 2.14	3.57**
190	11.68 ± 0.90	2.87**	24.02 ± 2.06	3.83**
191	11.26 ± 0.75	2.26*	23.81 ± 2.17	5.45**
194	11.97 ± 1.01	2.67**	25.83 ± 2.23	3.90**
195	11.93 ± 0.97	2.57**	25.47 ± 2.19	7.71**
199	11.74 ± 0.95	1.22	24.33 ± 2.38	5.66**
201	12.08 ± 4.75	0.87	23.95 ± 1.91	2.85**
200	12.24 ± 4.59	1.86	23.15 ± 1.88	3.97**
203	11.38 ± 0.86	1.82	23.56 ± 2.26	3.19**
202	11.61 ± 0.92	1.82	24.97 ± 2.02	3.26**
204	11.65 ± 1.03	1.70	24.06 ± 2.15	4.05**
207	11.57 ± 0.96	1.58	25.29 ± 2.25	4.12**
208	11.59 ± 0.97	2.54**	25.24 ± 2.32	5.59**
17	11.79 ± 1.03	1.74	23.46 ± 2.13	1.88
12	11.66 ± 1.11	2.13*	22.89 ± 2.14	3.60**
	Width		Length	
Grand mean	11.69 ± 1.54		24.42 ± 2.60	
Observed *F* values:				
Between isolates	4.693**		8.57**	
Between colonies	12.039**		33.68**	
Expected *F* values:				
Between isolates	418/10,560	=	1.11:1.15	
Between colonies	21/418		1.60:1.91	
† Between colonies within isolates	19/480		1.90:2.51	

peared to be as great as or greater than that between spores from colonies of the separate isolates. Regression analysis to determine the correlation coefficient between width and length of spores in these isolates yielded an *R* value of .424, again indicating that width and length show a poor correlation. "*T*" tests showed that *R* is significantly different from zero, however, indicating some correlation, although low, between width and length in this sample.

Spore morphology

The poor correlation coefficients between width and length suggested that the spores may have some unique feature other than size and external ornamentation that would be useful for taxonomic differentiation. Additional measurements were therefore made of the length from the septum to either end of the spore, and also of the angle of curvature at the terminal ends of the spores (fig. 2). These observations were made over the same spores observed for spore width-length analysis. Table 7 shows figures representing two canes per region, five pycnidia per cane, and 33 observations per pycnidia. It gives the mean and standard deviation

TABLE 7

MEANS AND STANDARD DEVIATIONS OF *DIPLODIA NATALENSIS* SPORE ANGLES AND MORPHOLOGY FROM 10 DIFFERENT REGIONS. FIGURES REPRESENT TWO CANES PER REGION WITH FIVE PYCNIDIA PER CANE, AND 33 OBSERVATIONS PER PYCNIDIUM

Region	Variable A*	Variable B*	Variable α	Variable β
	Mean	Mean	Mean	Mean
3A	5.94 ± .708	7.50 ± .858	21.35 ± 6.143	33.30 ± 8.626
4A	5.85 ± .701	7.47 ± .909	19.55 ± 7.242	34.39 ± 8.012
6A	6.09 ± .760	7.62 ± .881	21.74 ± 8.359	32.97 ± 8.780
12A	5.90 ± .814	7.73 ± .912	18.69 ± 6.882	33.72 ± 8.217
17A	6.17 ± .732	7.70 ± .842	21.90 ± 7.135	31.96 ± 8.913
24A	6.08 ± .752	7.69 ± .912	17.17 ± 5.908	31.22 ± 8.155
26A	5.77 ± .694	7.25 ± .728	18.33 ± 6.600	27.06 ± 8.707
27A	5.83 ± .615	7.20 ± .789	21.21 ± 7.867	32.16 ± 9.308
30A	5.79 ± .735	7.76 ± 1.027	17.08 ± 6.496	34.56 ± 8.859
34A	5.80 ± .763	7.54 ± .892	20.64 ± 6.282	33.33 ± 8.700
Observed F values:				
Pycnidia	5.115	5.324	13.473	6.076
Canes	1.487	2.052	1.446	2.716
Regions	1.882	1.712	1.651	1.479

Required F values: pycnidia, 1.28 (.05) and 1.41 (.01); regions, 3.02 (.05) and 4.95 (.01).

* Values for A and B should be corrected by a factor of 1.6 to compensate for units used in making observations. Values represent degrees of the angles.

for the variables A, B, α, and β (fig. 2), along with F values calculated to determine variation between regions, canes, and pycnidia.

Table 8 gives F values obtained in an analysis of variance between the regions, canes, or pycnidia. The table shows that the curvature angles of the spores may be as variable as width and length, as is also the case with the variables A and B. Correlation coefficients between A and B were determined, with R equal to .2046. The correlation coefficient, R, between α and β was .08, indicating a very poor correlation between the angles α and β.

DISCUSSION

We consider that results of the present study clearly indicate the nonvalidity of the presence or absence of stromata, grouped or scattered pycnidia, hirsute or glabrous, beaked or nonbeaked pycnidia, and the production of conidia, with or without mucus, as reliable taxonomic criteria in delimiting genera and species of the Phaeodidymous Sphaeropsidales as a whole. In contrast, the consistent observations of striate spores, the presence of paraphyses, and spore size in all of the collections studied indicate the potential of increased emphasis of these characters in future taxonomic considerations of these fungi. For example, Zambettakis (1953, 1954) erected the genus *Strionemadiplodia* to include members of the Phaeodidymous Sphaeropsidales with striate spores and paraphyses present in glabrous, separate pycnidia. He maintained that *Lasiodiplodia* Griffon and Maublanc (*sensu nobis*) was distinguished from *Strionemadiplodia* by having pycnidia that are "hairy" and produced in groups. Both genera are characterized by striate pycnidiospores and paraphyses in the pycnidium. *Diplodia frumenti* Ell. &

TABLE 8

F VALUES OBTAINED FROM AN ANALYSIS OF VARIANCE TO DETERMINE VARIATION AMONG VARIABLES A, B, α, AND β WHERE *DIPLODIA NATALENSIS* PYCNIDIA ARE NESTED WITHIN THOMPSON SEEDLESS CANES, AND CANES WITHIN REGIONS

Region	*F* values obtained among:											
	Canes within region				Pycnidia within region				Pycnidia within cane			
	A	B	α	β	A	B	α	β	A	B	α	β
3A	0.836	0.433	5.091	0.072	0.716	0.944	0.877	2.308				
Cane 1									0.256	0.900	1.552	2.333
Cane 2									1.158	0.997	0.356	2.280
4A	6.101	14.961	3.273	0.588	3.715	1.353	22.964	7.127				
Cane 1									3.947	1.060	4.901	0.666
Cane 2									3.486	1.639	58.860	18.140
6A	2.021	0.357	0.044	0.109	2.273	5.930	36.504	5.411				
Cane 1									1.265	4.705	64.353	8.140
Cane 2									4.216	7.362	6.152	3.513
12A	0.043	0.608	2.712	8.335	20.02	8.805	13.399	5.352				
Cane 1									24.40	5.585	1.445	3.158
Cane 2									15.88	11.473	32.075	7.450
17A	1.626	0.136	2.964	6.075	4.726	4.050	2.524	3.234				
Cane 1									6.529	2.991	2.812	6.066
Cane 2									3.157	4.916	2.184	0.657
24A	1.196	0.988	3.635	14.207	5.477	14.798	16.238	4.759				
Cane 1									4.887	5.891	31.675	5.721
Cane 2									6.208	21.730	3.817	3.914
26A	0.374	0.048	0.0312	0.914	6.964	5.578	8.556	9.763				
Cane 1									2.130	2.030	15.823	16.586
Cane 2									11.854	8.735	1.179	3.747
27A	4.195	8.514	0.402	2.165	2.490	3.170	21.761	10.700				
Cane 1									3.941	0.313	25.336	11.989
Cane 2									0.727	7.411	16.604	9.413
30A	2.908	3.473	2.695	1.176	4.582	8.988	15.072	11.674				
Cane 1									3.313	14.694	35.425	13.767
Cane 2									6.011	3.710	4.059	10.608
34A	5.194	3.889	0.208	0.002	1.665	1.078	2.696	2.029				
Cane 1									1.529	0.479	3.313	3.044
Cane 2									1.802	1.700	1.873	0.945
Required *F* values:												
.01			5.32		1.97		2.43					
.05			11.26		2.57		3.44					

Ev. was transferred to the genus *Strionemadiplodia* as the type *S. frumenti* (Ell. & Ev.) Zamb. Zambettakis (1953, 1954) considered *S. frumenti* and *D. natalensis* P. Evans as synonyms. *Striodiplodia* Zamb. is distinguished from *Strionemadiplodia* Zamb. by the absence of paraphyses. Both genera contain species with striate spores.

Lasiodiplodia (Clendenin, 1896), as founded by Ellis and Everhart in 1896 was characterized by pycnidia and subicle (dense felt of hyphae covering the substratum from which the fruit bodies arise) enclosed in a hemispherical stroma. The paraphyses are intermingled with the conidiophores in the pycnidia, and otherwise as in *Diplodia*.

Diplodia Fr. (Fries, 1849) was founded in 1849 and characterized at that time 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." The genus *Diplodia* was maintained *in sensu nobis* by Zambettakis (1953, 1954) and reserved for species with smooth spores, paraphyses and stromata absent, and separate, ostiolated, smooth (glabrous), fleshy-walled pycnidia without necks.

The genus *Botryodiplodia* was founded by Saccardo (1880) and was characterized by dark, one-septate spores and pycnidia caespitose (aggregated in tufts but not grown together or fused; clustered, crowded), erumpent, and in a stroma; hairy or hairless. Zambettakis (1953, 1954) differentiates *Botryodiplodia* on the basis of smooth, dark, two-celled spores, the absence of paraphyses, and at maturity stromata present with individual pycnidia with membranous walls.

Goos, Cox, and Stotzky (1961) reviewed literature on the host range and taxonomy of the species *Botryodiplodia theobromae* Pat. They also observed considerable overlapping in characters previously used to delimit genera, as did Wardlaw (1932). Both Shear (1933) and N. Stevens (1941) believed that *D. natalensis* P. Evans and *B. theobromae* are identical. Zambettakis (1953, 1954) placed this fungus in *Lasiodiplodia* Gr. & Maubl., with *L. theobromae* (Pat.) Gr. & Maubl. as the type of the genus. He did not consider it a synonym of *S. frumenti.*

The genus *Diplodiella* Karst. (1884*a*) was described as being the same as *Diplodia* Fr., but with scattered pycnidia with bristles or hairs. Zambettakis (1953, 1954) retained these genera and distinguished them as having dark, smooth, two-celled spores, no stromata, and ostiolate pycnidia. According to him, they differ in that *Diplodiella* has smooth, superficial pycnidia whereas the pycnidia of *Chaetodiplodia* Karst. (1884*b*) are hairy and not beaked. *Chaetodiplodia* is identical with yet another genus, *Rhynchodiplodia* Briosi & Farneti, except that the pycnidia of the latter are beaked (Zambettakis, 1953, 1954).

Bender (1934) recognized all of the above general except *Strionemadiplodia* and *Striodiplodia,* which were founded after the appearance of his key. That work emphasized the distribution of pycnidia, presence or absence and nature of the stromata, and pycnidial characters. Bender also recognized as distinguishing generic characters the conidiophore characters, relation of pycnidium to substratum, and production of conidia in or without mucus.

Griffon and Maublanc (1909) also recognized *Diplodia* Fr., *Rhynchodiplodia* Br. & Farn., *Chaetodiplodia* Karst., *Pellionella* Sacc., *Diplodiella* Karst., *Botryodiplodia* Sacc., and *Lasiodiplodia* Ell. & Ev. Characters utilized by them in generic separation included: grouping of pycnidia; stromata present or absent; relation of pycnidia to substratum; stromata glabrous or hairy; presence or absence of paraphyses; pycnidia glabrous or hairy; spore size; presence or absence of mucus; and pycnidia beaked or not. Taubenhaus (1915) studied cultures of *L. tubericola* Ell. & Ev., *L. theobromae* (Pat.) Griff. & Maubl., *D. gossypii* Zim, and *D. natalensis* P. Evans, and suggested that of the genera *Diplodia* Fr., *Botryodiplodia* Sacc., *Diplodiella* Karst., *Chaetodiplodia* Karst., and *Lasiodiplodia* Ell. & Ev., only *Diplodia* is valid.

N. E. Stevens and Wilcox (1925) and N. E. Stevens (1926) reported that *D. natalensis* is an imperfect state of *Physalospora rhodina* (Berk. & Curt). Voorhees (1942) listed *P. rhodina* and *B. theobromae* as synonyms. At the same time, Voorhees noted that "there are at least three distinct species of *Physalo-*

spora that are not distinguished by their *Diplodia* states. Thus it is entirely possible that many of the *Diplodia* forms included under *P. rhodina* have their sexual stages in undescribed species of *Physalospora* and other genera." Thus, an attempt to delimit species of this group, on the basis of their genetic relationship, to perfect stages quite possibly would result in difficulties similar to those encountered with the imperfect forms.

In the present study, single-spore colonies from a single pycnidium displayed most of the above-mentioned characters, and all were observed in single-spore colonies from pycnidia from a single cane. We believe that all of the isolates studied from grape belong to the species *D. natalensis* P. Evans (*Strionemadiplodia frumenti* Zamb.). This conclusion is based on the fact that all single-spore colonies studied produced spores that were characteristically striated, and most of the pycnidia were observed to produce paraphyses. The validity of certain characters (i.e., paraphyses, striate spores, and spore size) for use in identifying this fungus is further substantiated by the fact that they were constant both in culture and on canes collected from the field. This was also found to be the case when corn leaves and pea straw were used as substrates, and it suggests that comparisons of species recorded in the literature from an array of hosts could be made strictly on the basis of the presence of paraphyses and spore morphology. The failure to observe paraphyses in some of the pycnidia is considered to be due to the age of the pycnidium at the time of observation. Thus we agree with Zambettakis (1953, 1954) that the ornamentation of the spore and the presence or absence of paraphyses should be given prime importance in distinguishing members of this group. On the other hand, our results do not agree with maintenance of the genus *Lasiodiplodia,* since it differs from *Strionemadiplodia* only in that the latter contains species with grouped, hairy pycnidia, whereas the former contains species with glabrous, separate pycnidia. Furthermore, if we accept the concept of the genus *Diplodia* as offered by Taubenhaus (1915), the only remaining valid character for differentiation would be ornamentation of the spore walls, since the other characters recognized in the literature at present have been proved subject to extreme variation resulting from substrate and environment (Goos, *et al.,* 1961; Satour *et al.,* first and second papers; Taubenhaus, 1915; Wardlaw, 1932). This finding is further substantiated by the inherent variability revealed in the present study, when large numbers of single-spore colonies were compared under standard conditions.

Since the range of spore size observed in the present study is consistent with that reported by others (Evans, 1910; N. E. Stevens, 1926; Verall, 1942) for *D. natalensis,* it is not suggested that the statistically significant differences observed in spore size imply a basis for considering any of our isolates as separate strains. The results, however, point out the necessity for comparing the variation found in a single pycnidium with that from several other collections so that a better estimate can be made of the range of variation that a particular species may be capable of producing.

The differences in colony types observed are interesting not only from the standpoint of stromata and aerial mycelium production but also as they may relate to the possibility of identifying different strains. Hansen (1938), in his paper on the dual phenomenon in imperfect fungi, found that the dual phenomenon appears to occur more frequently in isolates from the Sphaeropsidales and Melanconiales than in the Moniliales. He designated three cul-

ture types: mycelial; conidial; and intermediate. In our observations, no sterile colonies were found in more than 3,000 single-spore colonies, although numbers of pycnidia ranged from very few to many, as shown in the figures. The fact that distinctly different colony types were observed in single-spore series from a large number of the individual pycnidia analyzed indicates either a heterocaryotic condition or that pycnidia may be composed of hyphal elements from more than one strain infecting the same cane.

Our results indicate that all of the collections studied are representatives of *D. natalensis* and that many of the characters normally considered to delimit the species may be more variable than previously thought. Spore size, morphology, ornamentation and presence or absence of paraphyses in the pycnidia are the most reliable characters for distinguishing this species. We do not wish to imply that the problem of identifying fungi of this group has a simple and immediate answer. We do believe, however, that a better consideration of the variability that these organisms are capable of would help eliminate considerable confusion. On that basis, and in view of the results of the present study, we are now analyzing over 250 isolates representing numerous previously described genera and species of the Phaeodidymous Sphaeropsidales. Results, together with suggested taxonomic revisions, will be published elsewhere.

LITERATURE CITED

BARBE, G. DOUGLAS, AND W. B. HEWITT
1965. The principal fungus in the summer bunch rot of grapes. Phytopathology **55**: 815–16.
BENDER, H. B.
1934. Key to the genera Phaeodidymous Sphaeropsidales. Privately published. New Haven, Conn.: The Tuttle, Morehouse & Taylor Co., 52 pp.
CLENDENIN, IDA
1896. *Lasiodiplodia* E. & E. Bot. Gaz. **21**: 92.
EVANS, I. B. P.
1910. On the structure and life history of *Diplodia natalensis* n. sp. Transvaal Dept. Agr. Sci. Bul. **4**: 18 pp.
FRIES, E.
1846–1849. 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.
GRIFFON, W. M., AND A. MAUBLANC
1909. Sur une maladie du cacaoyer. Bul. Soc. Mycol. France **25**: 51–58.
GROVE, W. B.
1937. British stem and leaf fungi. Vol. 2 (Coelomycetes). London: Cambridge Univ. Press, 406 pp.
HANSEN, H. N.
1938. The dual phenomenon in imperfect fungi. Mycologia **30**: 442–55.
HEWITT, W. B., G. V. GOODING, Jr., L. CHIARAPPA, AND E. E. BUTLER
1962. Etiology of summer bunch rot of grapes in California. Phytopathology **52**: 13. (Abstract)
KARSTEN, P. A.
1884*a*. Diplodiella. Hedwigia **23**: 62.
1884*b*. Fragmenta mycologia **16**: 62.
SACCARDO, P.
1880. Michelia **2**: 7.
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.
STEVENS, N. E.
1926. Two species of *Physalospora* on citrus and other hosts. Mycologia **18**: 206–17.
STEVENS, N. E., AND MARGUERITE S. WILCOX
1925. The citrus stem end rot *Diplodia* and its life history and relation to *Sphaeropsis malorum*. Phytopathology **15**: 332–40.
TAUBENHAUS, J. J.
1915. Notes on the probable non-validity of some genera of Phaeodidymous Sphaeropsidales. Amer. Jour. Bot. **2**: 324–31.
VERALL, A. F.
1942. A comparison of *Diplodia natalensis* from stained wood and other sources. Phytopathology **32**: 879–84.
VOORHEES, R. K.
1942. Life history and taxonomy of the fungus *Physalospora rhodina*. Florida Agr. Exp. Sta. Bul. **371**.
WARDLAW, C. W.
1932. Observations on the pycnidium of *Botryodiplodia theobromae* Pat. Ann. Bot. **46**: 229–38.
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.

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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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