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CONTENTS

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SPORIDIAL FUSION IN USTILAGO MAYDIS [1]

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INTRODUCTION

Cytological and cultural investigations of *Ustilago maydis* (DC.) Cda.[3] reported in the literature have left certain phases of the knowledge concerning sporidial fusion and nuclear phenomena in this species in a state of controversy. Maire (*10*) [4] in 1898 suggested that fusion of sporidia occurred in culture. He showed one illustration of fusion but did not describe the nuclear condition. Later Sartoris (*14*) reported the occurrence of sporidial fusion in culture but did not describe or illustrate the process. Seyfert (*15*) was unable to confirm the work of Sartoris. Rawitscher (*11*) reported that the sporidia neither fused nor formed true mycelium but remained uninucleate. Hanna (*4, p. 430*) studied the physiology and cytology of sporidia in *U. zeae* and *Sorosporium reilianum* (Kuehn) McAlp. and concluded that ". . . it seems clear that the conditions which stimulate this process in many other smuts are without effect on the sporidia of *U. zeae*." Christensen (*2*) found it necessary to inoculate the host plant with paired cultures in order to study segregation of sex factors. Sleumer (*16*) obtained sporidial fusion in culture between compatible sporidia in *U. zeae*. His descriptions and illustrations of the process agree essentially with those given for other members of the Ustilaginaceae.

In view of the conflicting and incomplete information contained in the literature, the work reported herein was undertaken to determine (1) conditions conducive to sporidial fusion in culture and (2) the nuclear behavior following fusion.

MATERIAL AND METHODS

The chlamydospore material of *Ustilago maydis* used in this investigation was collected from inbred lines of corn (*Zea mays* L.) grown at Madison, Wis. Sporidial cultures of monobasidiospore (monosporidial) origin were used and were established by taking single basidio-

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[2] Grateful acknowledgment is due Dr. J. G. Dickson for helpful suggestions and criticisms throughout the course of this investigation.

[3] Its synonym, *Ustilago zeae* (Beckm.) Unger, is used in many articles cited.

[4] Italic numbers in parentheses refer to Literature Cited, p. 242.

spores from the basidium (promycelium) by the method described by Hanna (*3*).

Stock cultures were maintained on potato-dextrose agar. In the sporidial studies 1-percent plain, refined agar medium or dilute malt-extract liquid medium was used. A malt-extract stock solution was prepared from freshly ground malt used at the rate of 10 gm. per 100 cc. of distilled water. The extraction was carried out at temperatures of 40°, 50°, and 65° C. for successive periods of 10, 20, and 30 minutes, respectively. The liquid was then decanted and autoclaved. A subsequent filtration to remove the precipitate occasioned by autoclaving was necessary if a clear liquid was desired. This stock solution was used at the rate of 1 part to 100 parts of distilled water, unless otherwise stated.

Cultures of paired monosporidial lines were prepared for study on ordinary glass slides or as hanging-drop cultures on deep-well slides. Sporidia from 3- to 4-day-old potato-dextrose agar slants were used throughout these studies.

RESULTS

PHYSIOLOGY OF SPORIDIAL PRODUCTION AND FUSION

Sporidial fusion in certain of the Ustilaginaceae has been reported to be influenced by the nutrient concentration and reaction of the culture media (*5*, *6*, *14*). Sleumer (*16*) observed that sporidial fusion in *Ustilago zeae* occurred on 3-percent maltose or glucose media only after the nutrients were spent. However, he reported fusion also on malt-extract medium after 24 hours. He believed that fusion was most likely to occur when the reaction of the medium was pH 8.0 to 8.5. Kernkamp (*7*) found three growth types in *U. zeae:* (1) A sporidial type, (2) a mycelial type, and (3) different intergrades of an intermediate type. The growth type used in the present studies obviously was of the intermediate type in that it produced both sporidia and mycelium. Several preliminary tests were conducted with a mixture of compatible monosporidial cultures that were known to cause gall formation when inoculated into corn plants. These tests indicated that (1) the development of haploid hyphae by the sporidia and (2) the fusion of compatible sporidia occurred only in cultures of low nutritive value. Only vegetative budding of the sporidia was observed in cultures with nutrient concentrations common in many laboratory media. These findings are in agreement with those of Kernkamp (*7*), who concluded that increasing the quantity of various nutrients, especially certain sugars, increased the sporidial growth in cultures of the intermediate type. He reported also that some cultures of the intermediate type were predominantly sporidial in nature when grown in solutions of relatively high nutritive concentration. However, as the cultures aged and the supply of nutrients decreased, the cultures became more mycelial in nature.

Additional tests were made with the same monobasidiospore lines in hanging-drop cultures in sterile distilled water and in malt extracts of 1 and 2 percent. After sterilization, the reaction of distilled water ranged from pH 7.0 to 7.2 and that of the malt extracts from pH 6.8 to 7.0. These cultures were incubated at various temperatures, of 4° intervals, from 4° to 36° C. and were examined after 24, 48, and 72 hours. Each test was made in duplicate and repeated once. The average results of the tests are given in table 1.

At the end of 24 hours, haploid hyphae and fused sporidia were evident in the distilled-water cultures incubated at temperatures of 20° C. or above. Sporidial production and growth rather than fusion appeared to be stimulated slightly in the 1-percent malt-extract cultures. At the end of 48 hours, in the 2-percent malt-extract cultures, the fewest haploid hyphae occurred and there were no sporidial fusions at any temperature. At the end of 72 hours, the percentages of haploid hyphae and sporidial fusions in the 2-percent malt-extract cultures had increased slightly, except at the lowest temperatures, but the percentages were still the lowest. In the cultures in all three media sporidial fusions were found to increase with advancing age. At two temperatures, approximately 6 percent of the sporidia in the distilled-water cultures had fused at the end of 72 hours.

TABLE 1.—*Influence of time, temperature, and culture media on the development of sporidia, haploid hyphae, and fusion pairs in Ustilago maydis*

Time (hours)	Temperature	Distilled water				1-percent malt extract				2-percent malt extract			
		Sporidia		Haploid hyphae [2]	Fusion pairs [2]	Sporidia		Haploid hyphae [2]	Fusion pairs [2]	Sporidia		Haploid hyphae [2]	Fusion pairs [2]
		Production [1]	Length [1]			Production [1 2]	Length [1]			Production [1]	Length [1]		
	° C.			*Percent*	*Percent*			*Percent*	*Percent*			*Percent*	*Percent*
24	36	2	2	25	T	2	2	10	0	3	3	0	0
	32	2	2	25	T	2	2	10–12	T	3	3	0	0
	28	2	2	20	T–1	2	2	10–12	T	3	2	0	0
	24	1	2	15	T–1	2	2	10	T	3	2	0	0
	20	1	1	10	T	2	2	5	0	3	2	0	0
	16	0	1	5	0	1	1	1–2	0	2	2	0	0
	12	0	1	0	0	1	1	0	0	1	1	0	0
	8	0	1	0	0	T	1	0	0	1	1	0	0
	4	0	1	0	0	T	1	0	0	1	1	0	0
48	36	2	3	40	------	3	4	20	------	4	4	3	0
	32	2	3	40	------	3	3	20	2–5	4	4	3	0
	28	2	2	30	3	3	3	20	2–5	4	3	2	0
	24	2	2	25	3–4	2	2	15	2	4	3	1–2	0
	20	1	1	15	3	2	2	10	1	3	2	T	0
	16	1	1	5	T–1	2	2	5	0	3	2	0	0
	12	1	1	------	0	1	1	T	0	2	1	0	0
	8	1	1	0	0	1	1	0	0	1	1	0	0
	4	1	1	0	0	1	1	0	0	1	1	0	0
72	36	2	3	40	------	3	4	30	------	4	4	5	T
	32	2	3	40	------	3	3	30	3	4	4	5	T
	28	2	2	30	4–6	3	3	30	3	4	4	3	T
	24	2	2	30	6	3	2	25	3	4	3	3	1
	20	2	2	25	3–4	2	2	20	1	3	3	1	T
	16	1	1	10	T	2	2	10	T	3	2	T	0
	12	1	1	T	0	1	1	T	0	2	1	0	0
	8	1	1	T	0	1	1	0	0	1	1	0	0
	4	1	1	0	0	1	1	0	0	1	1	0	0

[1] 0=No increase; 4=maximum increase; based on mean values.
[2] T=Trace.

The temperatures at which the cultures were incubated also influenced the incidence of sporidial fusion. Sporidia produced at the higher temperatures, 28°, 32°, and 36° C., were long and narrow. This condition, together with the rapid multiplication of sporidia by vegetative budding at these temperatures, made the identification of haploid hyphae and fusion pairs difficult and uncertain. In general, a decrease in temperature was accompanied by decreases in the number and size of sporidia, in the number and length of haploid hyphae,

and in the prevalence of fused sporidia. No sporidial fusion and only a small amount of growth occurred in the cultures held at 12° or lower for 72 hours. These cultures and those incubated at the lower temperatures were continued for an additional 7-day period. At that time a few fused sporidia were observed both in the distilled-water and in the 1-percent malt-extract cultures incubated at 12°. No fusion was observed at the lower temperatures.

For a closer study of sporidial fusion, cultures were prepared from paired sporidial lines of monobasidiospore origin in sterile distilled water and in 1-percent malt-extract solutions. These cultures were incubated at 20° and 24° C. Fusion was observed first after 15 and 20 hours in the malt-extract cultures incubated at 24° and 20°, respectively, and after 20 hours in the distilled-water cultures incubated at 24°. Fusion pairs were not abundant, however, until the cultures were 40 to 48 hours old. This confirms the work of Sleumer (*16*) with

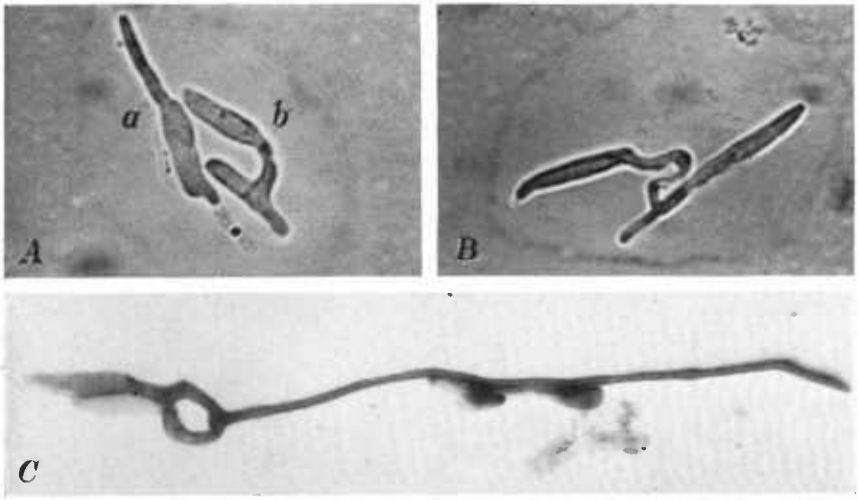

FIGURE 1.—Sporidial fusion in *Ustilago maydis*. Approximately ×1,200. *A*, *a*, Sporidium germinating, with uninucleate hyphae developing from both ends of the spore; *b*, an early stage of fusion between two compatible sporidia, with the binucleate hypha developing from the end cell of one sporidium. *B*, An early stage of fusion between sporidia, similar to that in *A*, *b*. *C*, A later stage in sporidial fusion, in which the fusion hypha is several cells long. *A* and *B* were stained with acid fuchsin and *C* with Delafield's haematoxylin.

the same fungus in respect to time required for fusion to take place. The fusion process between compatible sporidia of *Ustilago maydis* differs only slightly from that reported for certain other smuts. The conjugation process did not seem to follow any definite pattern such as that described by Holton (*6*) for *U. avenae* and *U. levis*. Neither sporidium appeared to take a more active part than the other. Fusion appeared to fall into three general types: (1) Direct fusion of two adjacent sporidia; (2) fusion of young one- or two-celled hyphae (germ tubes) formed from sporidia close to each other; and (3) fusion of cells of two older uninucleate mycelia. The uninucleate hyphae by means of which the fusions occurred were found to vary considerably in diameter and length. Representative stages of fusion are

shown in figure 1. In figure 1, *A*, *a*, uninucleate hyphae have developed from both cells of a two-celled, nonfused sporidium. Early stages of fusion are illustrated in figure 1, *A*, *b*, and *B*. In each case the fusion hypha has just started its development. A somewhat later stage in which the fusion hypha is several cells in length is shown in figure 1, *C*. In this case the sporidia have not fused directly although in juxtaposition, but rather fusion has taken place between the short hyphae developed from each.

Although fusion was more commonly observed between compatible sporidia close to each other, it was observed also between cells of uninucleate hyphae originating from widely separated sporidia. This type of fusion is not peculiar to *Ustilago maydis*, as a similar type of fusion has been reported by Stakman, Cassell, and Moore (*17*) for *Urocystis occulta* (Wallr.) Rab. In a few instances compatible sporidia that had apparently fused end to end by means of short germ tubes were observed. In such cases the binucleate hypha developed from the opposite end of one sporidium.

The hyphal outgrowth initiating the binucleate condition develops soon after the fusion of two compatible gametes. The hyphae grow rapidly and under favorable conditions often reach a considerable length. Occasionally it was possible to follow binucleate hyphae that had grown across the culture drop for distances of approximately 5 to 8 mm. (5,000μ to 8,000μ). Sleumer (*16*) observed one such hypha that had reached a length of 340μ.

Holton (*6*) and Harper (*5*) reported that the hyphae developed from fused sporidia in *Ustilago avenae* and *U. levis* and in *U. antherarum*, respectively, revert to the production of sporidia eventually. Sleumer (*16*) stated that the addition of nutrient material to cultures of *U. zeae* resulted in sporidial formation by both uninucleate and binucleate mycelia. The possible influence of adding nutrients to the cultures was not investigated by the writers. Hanging-drop cultures were maintained in the laboratory for 2 months, however, without any evidence of sporidial formation from binucleate hyphae. Frequently uninucleate hyphae were found to produce aerial sporidia at the surface of the culture drop. The binucleate hyphae varied considerably in size and often were no coarser than the uninucleate hyphae. Hence, it became necessary to trace any hypha in question to its origin or resort to staining procedure to determine its nuclear condition.

NUCLEAR BEHAVIOR AFTER FUSION

When sporidial cultures had reached the desired stage for staining, they were killed with Flemming's weaker solution or Carnoy's alcohol-acetic acid solution or simply by drying them rapidly over a low flame. When thoroughly dried, the cultures were found to adhere to the slide or cover slip sufficiently well to permit their manipulation in the staining procedure without appreciable loss.

The protoplasmic contents in the rapidly growing portions of both uninucleate and binucleate hyphae exhibited a marked affinity for certain dyes and destained very irregularly. This was especially true when crystal violet was used. Either Heidenhain's or Delafield's haematoxylin gave greater uniformity. The latter stain was more satisfactory because the cell walls of the preparations thus stained were more readily discernible. A weak concentration of acid fuchsin in

lactophenol was found to be fairly satisfactory as a general, semi-permanent, rapid stain.

The procedure followed with Heidenhain's iron alum haematoxylin was essentially the same as that described by Holton (*6*). The procedure employed with Delafield's haematoxylin was as follows: The preparation was dried, then fixed in dilute Carnoy's solution for 2 to 5 minutes; flooded with water, which was removed with filter paper or cotton swab; dried over a very low flame; stained 3 to 30 minutes; washed by flooding with water; destained in acid alcohol; transferred to 70-percent alcohol and dehydrated through the higher alcohols; and then cleared and mounted in Canada balsam.

The nuclear behavior and distribution of the protoplasmic contents of the fused sporidia and binucleate hyphae in *Ustilago maydis* were found to differ in some respects from the descriptions for other members of the Ustilaginaceae. Several investigators (*1*, *6*, *8*, *9*, *11*, *12*, *13*, *16*) agree essentially that after fusion the protoplasmic contents of the fused sporidia migrate into the fusion hypha and thereby initiate the binucleate phase. The contents then continue to move toward the apex as the hypha elongates. Sleumer (*16*), Rodenhiser (*13*), and Holton (*6*), investigating *U. zeae*, *Sphacelotheca sorghi* and *S. cruenta*, and *U. avenae* and *U. levis*, respectively, showed the formation of cross walls in the basal portions of old binucleate hyphae, although each of the cells thus formed, as well as the fused sporidia, apparently was empty. Stakman, Cassell, and Moore (*17*) found essentially the same situation in *Urocystis occulta*. However, they occasionally observed two-celled fused sporidia in which the nucleus was present in the nonfused cell.

In the present study, definite, well-defined nuclei were observed frequently in the fused sporidia and in the older basal portions of the binucleate hyphae, but their occurrence was more or less sporadic. Some cells both of sporidia and of hyphae apparently still contained the normal protoplasmic contents, whereas in others the contents were in various stages of disintegration and some cells appeared to be empty. The representative nuclear condition of the fused sporidia and older portions of the binucleate hyphae are illustrated in figure 2. In figure 2, *A*, the direct H-shaped type of fusion between two sporidia has just occurred. Both sporidia are two-celled. A single nucleus is evident in each of the fused cells and also in one of the nonfused cells. This last cell has produced a uninucleate hypha, which would make it possible for the one sporidium to fuse with two different sporidia or for a double fusion between two sporidia to occur.

It was possible to follow the entire binucleate hypha that had developed from the fused sporidia shown in figure 2, *D*. Representative portions of this hypha are shown in *E*, *F*, and *G*. The protoplasmic contents of the cells in *D* apparently were disintegrating, although scattered nuclei were clearly visible. In the fourth cell from the point of fusion (*E*, *a*) no nuclei were evident and only one each was observed in the fifth (*E*, *b*) and sixth (*E*, *c*) cells. The distribution of the cytoplasm in all three cells was rather irregular. In the middle portion of this hypha, the sixteenth cell (*F*, *b*) contained two clearly defined nuclei. Only one nucleus was visible in the seventeenth cell (*F*, *c*), and none was visible in either the fifteenth (*F*, *a*) or the eighteenth (*F*, *d*) cell. The cytoplasm, although not so uneven or patchy as in the older cells, was nevertheless in sharp contrast to the regular dis-

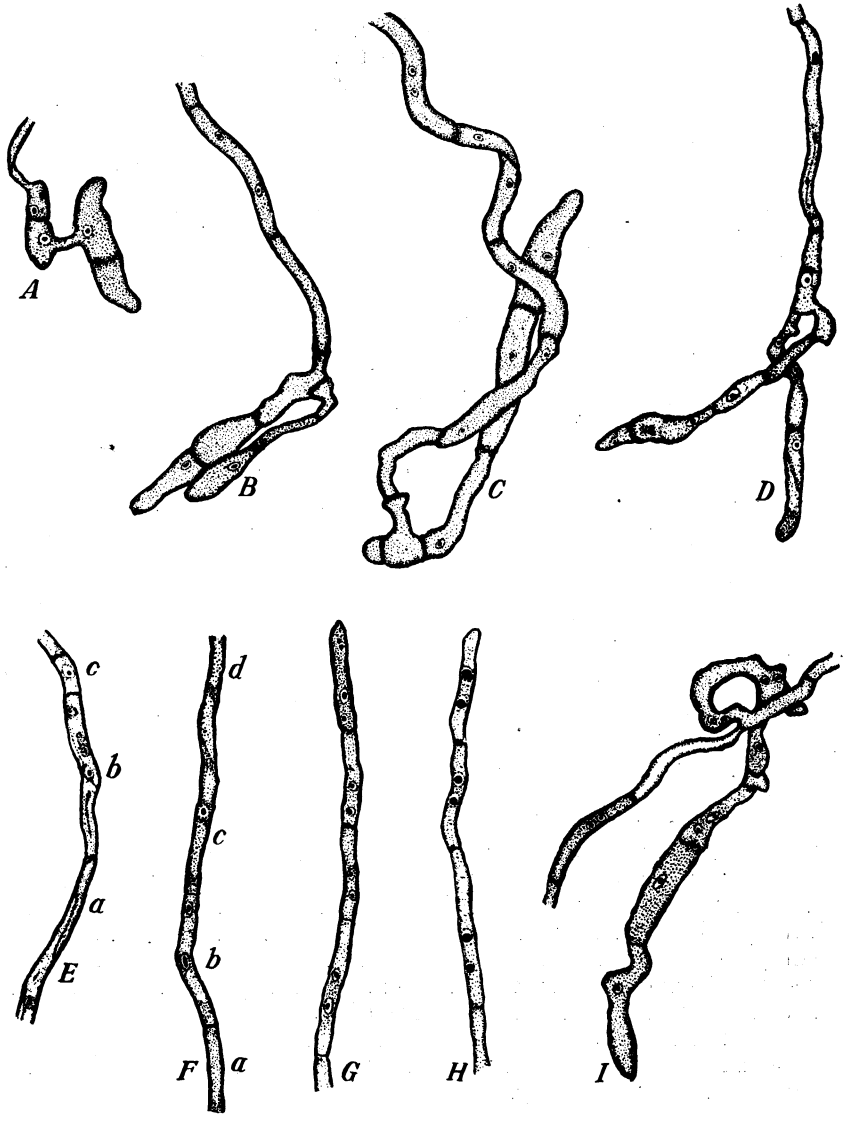

FIGURE 2.—Sporidial fusion and nuclear condition in *Ustilago maydis*. Approximately ×1,825. *A*, An early stage in the direct H-shaped type of sporidial fusion. Both sporidia are two-celled. The binucleate hypha has not yet developed. *B*, Sporidial fusion by means of short, uninucleate hyphae, or germ tubes, developed from ends of compatible sporidia. Paired nuclei are visible in the second cell of the binucleate hypha. *C*, Sporidial fusion in which one cell of one sporidium has functioned as the fusion cell. Paired nuclei are shown in three cells of the binucleate hypha. *D*, Fused sporidia. *E* to *G*, Representative portions of the binucleate hypha developed from the fused sporidia in *D*. The irregular cytoplasmic and nuclear condition commonly found in fused sporidia and older portions of binucleate hyphae is shown in *D*, *E*, and *F*. *G*, Paired nuclei in the tip cell of the hypha. *H*, Paired nuclei in the three apical cells of a representative binucleate hypha. *I*, Irregular and branched binucleate hypha. *A*, *B*, *C*, and *I*, stained with acid fuchsin; *D* to *G*, with Delafield's haematoxylin; *H*, with orseilline BB and aniline blue. (See text, p. 238, for explanations of *a*, *b*, *c*, and *d*.)

tribution of that in the younger cells near the tip of the hypha, as shown in *G*. There was also a decided difference in the staining reaction of the older and younger portions of the hypha. The older cells that contained one or more nuclei each, although staining more densely than those in which no nuclei were apparent, did not retain as much of the stain as did the rapidly growing cells at the end of the hypha.

Sleumer (*16*) distinguished three and sometimes four nuclei in the tips of binucleate hyphae in *Ustilago zeae* but did not show pairs of nuclei separated by cell walls. Allison (*1*), reporting on *U. levis* and *U. hordei* (Pers.) Lagerh., pictured one hypha containing six nuclei and another containing four, but no septa in either. Stakman, Cassell, and Moore (*17*) found that the binucleate hyphae of *Urocystis occulta* often contain two pairs of nuclei, frequently three nuclei, but rarely a single nucleus. They assume that the fourth and the second nucleus, respectively, may not have been visible or that one nucleus had not yet divided.

In the present study, paired nuclei, clearly delimited by septa, have been commonly observed in the end cells of binucleate hyphae. Paired nuclei were commonly found in the two end cells and occasionally in as many as five contiguous cells at the end of a binucleate hypha. Representative examples are illustrated in figure 2, *G* and *H*. In *G*, two small, darkly stained bodies are just visible in the tip of the apical cell. Whether these represent two daughter nuclei produced by division prior to cell-wall formation could not be definitely determined. The preceding five cells of this hypha (not illustrated) were well stained, but no pairs of nuclei could be definitely determined. The nucleoli shown in the three end cells of another binucleate hypha (*H*) appear to be slightly larger than those in other illustrations. This may have been due to the use of orseilline BB and aniline blue in place of Delafield's haematoxylin in the staining procedure.

Occasionally irregularly formed and branched binucleate hyphae (fig. 2, *I*) were observed. Sleumer (*16*) described a somewhat similar condition, which he termed "Wirkopulation." Inasmuch as the binucleate hyphae become branched and irregular in form within the host tissues, the occurrence of this condition in culture would seem to be more or less expected and not an exception in need of an explanation. One cell apparently contains two pairs of nuclei. The failure of a cell wall to form might have caused this condition; or possibly, as suggested by the shape of the cell, a new branch was beginning to develop into which the nuclei would migrate.

DISCUSSION

That the binucleate stage in the smut fungi is initiated in culture by the fusion of basidiospores (sporidia) has been demonstrated for a number of species in the Ustilaginaceae. However, the ability of the sporidia of *Ustilago maydis* to fuse in culture has not been clearly established previously. In the present study the fusion of compatible sporidia was obtained in culture. Its occurrence was found to depend largely on nutrition and temperature. Both factors influenced the time required for fusion to take place. In certain other smuts fusion occurs in a relatively short period of time. Holton (*6*) obtained fusion in *U. avenae* and *U. levis* after 35 minutes to 4 hours. Stakman,

Cassell, and Moore (*17*) observed fusion between the sporidia of *Urocystis occulta* as soon as the sporidia were fully formed. Rodenhiser (*13*) observed fusion in *Sphacelotheca sorghi* and *S. cruenta* after 10 hours. In the present study, however, fusion was first detected after 15 to 20 hours and was not readily observed until after 40 to 48 hours. The relatively long period of time and the extremely low concentration of nutrients, which apparently are necessary for sporidial fusion, may account for the negative results of many previous investigators.

From the present investigation of *Ustilago maydis* it could be deduced that the general nuclear behavior might be as follows. Simultaneously with, or immediately after, conjugation, the nucleus in each of the fusing gametes divides and a daughter nucleus migrates into the fusion tube or cell. Cell division is completed in the normal manner by means of cell-wall formation. Further growth of the binucleate hyphae is accomplished by normal cell division, i. e., nuclear division, migration of daughter nuclei, and cell-wall deposition. Each cell of the binucleate hyphae, therefore, normally contains one pair of nuclei.

The gradual aging and decline of the cells in the older portion of the hypha simultaneously with the growth of the apical region suggest that a disintegration of the protoplasmic contents of the older cells occurs at a rate depending upon the environmental conditions present in artificial culture. The extent to which this process is influenced by any particular set of environmental or nutritional conditions was not determined. The existence of physiological or biochemical differences between different portions of the hyphae is indicated by the differences in dye absorption and retention. Further studies might indicate a situation somewhat analogous to that described by Thomas (*18*) for several species of *Pythium*, in which he found that the fixation of dyes is determined by differences in cell-wall composition of young and that of old, mature hyphae. Whether the cells appear devoid of contents or contain nuclei either singly or in pairs would depend, therefore, upon the degree of protoplasmic aging and disintegration as well as upon the success of the staining technique.

If this interpretation is correct, the presence of empty fused sporidia and empty cells in old portions of binucleate hyphae or the appearance of cells with irregular numbers of nuclei might better be explained on the basis of physiological aging and disintegration of cell contents than of an irregular or unusual type of nuclear and cellular division. The viewpoint frequently expressed in the literature that the protoplasmic contents of fused sporidia pass into the fusion hypha and migrate toward the tip as growth occurs, leaving empty cells behind, was not found in this study to be applicable to *Ustilago maydis*.

SUMMARY

The production and fusion of sporidia in cultures of *Ustilago maydis* are described. The initiation of the binucleate stage by the fusion of compatible sporidia is described and illustrated. The fusion process was influenced markedly by the nutritive value of the culture media and by the incubation temperature.

Fused sporidia were sufficiently numerous to be readily detected after 20 hours at 24° C. in distilled water and after 15 to 20 hours at 20° to 24° in 1-percent malt-extract solution.

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The initiation of the binucleate phase followed no rigidly fixed method but resulted from the fusion of any two compatible haploid cells, either sporidial or hyphal. In no case were the binucleate hyphae observed to revert to sporidial production.

End cells of binucleate hyphae uniformly contained one pair of nuclei each. The protoplasmic contents of the older binucleate cells and of the fused sporidia appeared to be in various stages of disintegration. In some cases the cell contents were apparently normal, whereas in others they were either partly disintegrated or entirely lacking.

The rapidly growing apical cells of binucleate hyphae and young sporidia exhibited a pronounced affinity for the several dyes used, whereas older cells and old fused sporidia did not.

Since the results presented are based on a study of the fungus in culture they may or may not represent the situation as it exists in nature in the corn plant.

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