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ILLUSTRATIONS

THE NATURE OF SALTATION IN FUSARIUM AND HELMINTHOSPORIUM[1]

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INTRODUCTION

The phenomenon of saltation has been of interest to both geneticists and mycologists ever since it was first observed. A considerable amount of work has been done on the extent and on the causes underlying this phenomenon, but as yet there is very little real knowledge of its nature. By some authors, hyphal fusions, or anastamoses between adjacent hyphae, which are common in almost all fungi, have been considered to be a possible seat of the origin of saltation, in that such anastamoses provide a mechanism for the association or, perhaps, dissociation, of nuclei of different kinds within the same cell. As a consequence of this association, it is suggested that such a fusion cell may produce a new type. It is in this potential rôle of hyphal fusions that their genetic implication lies.

In the following pages, the experiments described have been designed to throw some light on the effects that these hyphal fusions produce, and on the conditions under which they are formed. The arrangement of the description of these experiments and their conclusions is as follows: In the first section are described some experiments and their evaluation are described which also strongly suggest that cyto- exist in the types used. In the second section, other experiments and their evaluation are described which also strongly suggest that cytoplasmic inheritance does not account for the differences between saltant strains and their parents in the types used. A general discussion follows, and in it the relation between these conclusions and the general phenomenon of saltation is pointed out.

Since the conclusions arrived at in these experiments are dependent partly upon negative evidence, it is as well to emphasize this fact, and to state that it is believed that the replication is sufficient to justify such conclusions.

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The author wishes to express his thanks to Dr. E. C. Stakman for his constant help and encouragement during these investigations, and to the Board of Regents of the University of Minnesota for the facilities provided.

Table 1

The Organisms Used, Their Source and Identification

Organism	Strain designation	From whom obtained	Date of isolation of original culture	By whom identified or described	Literature reference
Helminthosporium pedicellatum, Henry	HP	J. J. Christensen, University Farm, St. Paul, Minn.	1922	A. W. Henry (31)	Minn. Agr. Expt. Sta. Tech. Bull. 22:42-43. 1924
H. monoceros, Drec.	HM	Ditto	1920	C. Drechsler (24)	Jour. Agr. Res. 24:706-707. 1923
Helminthosporium sp. (Brachysporium type)	BN	Ditto	1922	J. J. Christensen	Not yet described
	BW	Isolated by author	1931		Saltant from BN.
Fusarium fructigenum, Fries.	FA_2	W. Brown, Imperial College of Science, London	1918	W. Brown (11)	Ann. Bot. 42:285-303. 1928
	$FB_{1.1}$	Ditto	1921	Ditto	Ditto
	$FB_{1.1\text{-}1}$	Isolated by author	1931		Saltant from $FB_{1.1}$
	$FB_{1.1\text{-}2}$	Ditto	1932		Saltant from $FB_{1.1}$
F. vasinfectum, Atk. var. *lutulatum*	FV	G. R. Bisby, Man. Agr. Col. Winnipeg, Man., Can.	?	C. D. Sherbakoff (43)	Cornell Univ. Agr. Expt. Sta. Memoir 6. 1915

ORGANISMS USED

The choice of organisms to be used in the sort of experiments made was of considerable importance. A list of the organisms used is given in Table 1, together with their origins and the authorities who named them and from whom they were obtained. The primary requirements for a suitable organism were considered to be the occurrence of numerous hyphal fusions, comparative facility for cultivation in the laboratory, the presence of non-air-borne spores, and a relative stability of type. In addition, to avoid too much exploratory work, it was decided to use organisms about which there existed some knowledge of their cultural characteristics. Also, it seemed desirable to choose organisms that produced saltations fairly freely; and for these reasons Helminthosporium and Fusarium were eventually selected for study.

REVIEW OF LITERATURE

Numerous observations on hyphal fusions, such as those of Stevens (46), Dosdall (23), and Christensen (15) on *Helminthosporium sativum,* are scattered through the literature; but such observations have been confined largely to reports of their occurrence in particular fungi. Further, the criterion used has usually been the growing together of two cells; the breaking down of the intervening wall has not been observed.

Laibach (33), one of the few workers who have studied hyphal fusions as hyphal fusions, found that no alteration in the frequency of their occurrence took place within the range of conditions he used. He did find, however, that of two closely allied species of Coniothyrium, *C. fuckelii,* and *C. concentricum,* the former showed numerous fusions; the latter showed none. From such observations, and from the fact that he obtained similar results with the fusing conidia of *Ustilago bromivora,* Laibach concluded that in both genera the fusions were vegetative in nature. On the other hand, Drechsler (24) reported that in his Helminthosporium strains more fusions tended to occur next to the glass bottom of the culture flasks. Brodie (7), however, concluded that fusions between the oidia of his *Coprinus lagopus* strains occurred more commonly under crowded conditions.

Matsumoto (36) has described fusions occurring between some but not all of his strains of Rhizoctonia spp. These fusions took place when the hyphae of two strains met while growing on an agar medium. Ezekiel (25) obtained apparently somewhat similar results with Sclerotinia spp.

Burgeff (13) was the first to suggest that hyphal fusions might result in mixochimaeras, the mixture of two or more strains. Stevens (46) and others have referred to this possibility, and Brierley (4, 5, 6)

has emphasized repeatedly this possible effect of hyphal fusions. At first Brierley (4) called the resultant condition of such a mixture "heterocaryosis," but later (5) he has returned to Burgeff's original term "mixochimaera." Heterocaryosis must, of course, be distinguished from heterozygosis.

Leonian (34) tried to form a mixochimaera between two strains of Fusarium by mixing inoculum from each on agar in a petri dish. At first he thought he had succeeded, but later he decided that his new form was a saltation from one of his parent cultures. Hansen and Smith (29) believed that they had been able to make new forms of Botrytis by a similar method.

Buller (12), in Volume 4 of his "Researches on Fungi," mentioned the observations of a number of the older workers who had noted the occurrence of hyphal fusions. He recalled Brefeld's observations (2) on *Coprinus stercorarius,* namely, the occurrence of numerous hyphal fusions in monosporous mycelia, and confirmed them from his own experience. In the case of Ascomycetes, Buller drew attention to the work of DeBary, Brefeld, the Tulasne brothers, and Woronin, all of whom mentioned and illustrated hyphal fusions in a number of species. Buller concluded that hyphal fusions were of common occurrence in both Basidiomycetes and Ascomycetes. On the other hand, in addition to confirming Zopf's illustration of hyphal fusions in *Syncephalis sp.,* Buller stated that he had been unable to find them in *Mucor mucedo, Pilobolus longipes,* and *Saprolegnia ferax;* therefore he considered that they are rare or absent in Phycomycetes.

Following this review of the older work, Buller pointed out that these hyphal fusions occurred in both haploid and diploid mycelium in Basidiomycetes, and that, in consequence, they can have nothing to do with sex. He also proceeded to quote examples of a social organization in many animals and plants, and developed the theme that these hyphal fusions were the mechanism for the development into one individual organism of a large number of hyphae, which may have arisen from different spores. Further, he drew attention to the exhaustion and consequent emptying of a mycelium, under starvation conditions, when only a part of it is in a suitable position to form spores (*Trichoderma lignorum*).

Considering this type of observation, Buller then enumerated five ways in which hyphal fusions were of importance in the Hymenomycetes: (1) for the conduction of food material, (2) for the mating of mycelia, (3) for the passage of nuclei through a haploid mycelium becoming diplodized, (4) for the preservation of unity of the mycelium in the event of mechanical injury, (5) for the social organization.

Recently, Davidson, Dowding, and Buller (18) have stated that they consider the presence of hyphal fusions in Dermatophytes a sign of similarity of species, and that only members of the same species will fuse with one another. They suggested further that this principle might be applicable both to Ascomycetes and Basidiomycetes.

EXPERIMENTAL SECTION I

HETEROCARYOSIS

A prime necessity in any genetical study of fungi, as Brierley (6) has emphasized, is to find out whether all the nuclei in the mycelium are of the same genetic constitution. For this purpose both cytological observations and cultural experiments are required. In the first part of this section a description of the development and nuclear condition of the strains dealt with is given; in the later part the cultural and isolation experiments are described.

A careful examination has been made of the development of the mycelium and the formation of the conidia in all the organisms used. For this purpose observations and series of drawings have been made on both living material and cytological preparations.

Methods

For the stages in the living material, cover slip cultures, both on liquid and on solid media, have been used, and no further comment is required on such technic. In the case of the cytological preparations a method has been used, which, altho it was originally devised in 1926 and has been in constant use since that time, has through an oversight not hitherto been described.

The cytological preparations were made from living material which had been growing on a microscope slide. The procedure consisted in first smearing a thin film of fresh egg albumen on a slide, and then flooding it with a suspension of fungal inoculum. After allowing the fungal material to settle for about a minute, the slide was drained, and put aside to dry. This drying process was found to be the critical part of the method. The slide must be allowed to dry in order to coagulate the egg albumen and so fix the inoculum to the slide, but it must not be allowed to dry sufficiently to kill the fungal material. In the case of thick-walled conidia, there was no difficulty; but in the case of pieces of hyphae or thin-walled conidia, some difficulty was found in determining when the drying process had gone far enough. As soon as the drying had been completed, the slide was placed across two strips of cork in a petri dish lined with moist filter paper. A suitable nutrient liquid was poured on the slide, and, if necessary, partially drained off.

The dish with the slide was then incubated at the appropriate temperature. Observations were made at suitable intervals and, when the required stage of development was reached, the slide was drained and plunged at once into a fixing solution. From this point the usual methods were employed.

For a fixative, Flemming's solution B diluted to two-thirds strength was found, if freshly made, to be satisfactory; but some of the best results were obtained by using glacial acetic acid and fixing for only two minutes.

The stains used have been Haidenhain's haematoxylin followed by a counter stain of Orange G, and the Feulgen "Nuclealreaktion" (35) with a counter stain of Jodgrün. The latter combination gave by far the best results, but, in the limited time available, it was impossible to determine the reason for failure to attain consistent success from month to month.

Formation of Conidia and Nuclear Content in Helminthosporium spp.

While the three species of Helminthosporium have all been examined, a description will be given only for *H. pedicellatum,* as the findings were similar in all three. Drawings of the critical stages are, however, given for all three species (see Figs. I, II).

The mycelium generally consisted of multinucleate segments making up the hyphae (Fig. I. 18, 19). Numerous hyphal fusions were found (Fig. II. 25, 26) more especially in the older parts of the colonies; and these anastamoses converted the hyphae into a mycelial network. Some of the cells showed unusual forms, swellings, etc. (Fig. I. 19); but such cells usually had a normal complement of nuclei. The number of nuclei in each cell varied from 1 or 2 to 8 or even more, and, as a rule, the young germ tube of a conidium had more nuclei in each cell than either the young growing hypha or the mature hypha of a colony (compare Figs II. 24; I. 8, 9; I. 18, 19). The apical cell of the young vegetative hypha (aerial or otherwise) had as a rule from 4 to 6 nuclei in it.

The young conidiophore, except when it was very young, could be recognized by its direction of growth being perpendicular to the surface of the medium, by its somewhat more swollen end as compared to the more blunt end of a vegetative hypha (see Figs. I. 8-16; II. 27-30, 34-36), and sometimes by a slight brownish tint coloring its wall. Using such criteria in the less mature parts of a colony, it was possible to find all stages from almost the first beginning of a conidiophore after it had turned upward, through its development of greater or less length of hypha, until the apical cell had begun to form spores. Such a series

is illustrated in Figs. I. 8-16; II. 27-30; 34-36. On the other hand, the vegetative hyphae, both aerial and otherwise, were invariably found to be without any suggestion of a swelling at their apices (Figs. I. 8-11; II. 27, 28, 34). This swelling of the young conidiophore tip was most marked in *H. pedicellatum* and least in *H. monoceros* (compare Figs. I. 14; II. 30). In consequence, the young conidiophore in *H. monoceros* is often difficult to recognize with certainty.

Stevens (46), it will be noted, found a binucleate or multinucleate condition in the mycelium. In the author's preparations, however, a few cells, nearly always the beginning of branches from older hyphae, contained one nucleus (Fig. I. 18, 19). It may be that such branches were the first stages in the formation of conidiophores, but this could not be determined definitely. What was certain, however, was that all hyphae with slightly swollen ends, which appeared to be young conidiophores, had a uninucleate apical cell in the earlier stages (Figs. I. 12, 13; II. 29, 35). In somewhat later stages the apical cell contained two nuclei (Figs. I. 14, 15; II. 30, 36, 37), while in other cases these two nuclei were clearly the products of one nuclear division (Fig. I. 14).

The mature conidiophore (Figs. I. 16; II. 31) has usually about two to four nuclei about the time the conidium begins to form. The formation of the conidium, as Ravn (41) has described, started as a fine "pin head," like an exudation drop, toward or at the apex of the conidiophore (Figs. I. 17; II. 31, 37). By the time the conidium was half grown, it had from five to ten nuclei. At this time the contents of the conidium, excepting the nuclei, still appeared to be homogeneous. It can not be stated definitely that nuclear division occurred in the conidium, but in view of the numbers sometimes found (100 or more) this seemed very probable. Soon after the conidium was half grown, the outer part of the conidial contents, toward the middle of the conidium, started to become more transparent and less granular than the original cytoplasm (Fig. II. 22, 23, 32). This appeared to be the formation of the "endosporium," and was due apparently to a change in the cytoplasm, and not to an ingrowth of the conidial wall. Stevens (46), following DeBary, believed this "endosporium" to represent morphologically a second cell wall. Nuclei have never been found in this lighter area. Later the segments in the conidium were delimited by ingrowths from the inner edge of the lighter area. The transverse septa have, in the center, a dot-like thickening—as Stevens (46) has figured it—which sometimes appeared to be similar to a pore.

The almost mature conidium may contain from one to six or eight, or even more, segments (Fig. II. 24, 32, 39). While it may fall from the conidiophore at almost any time after its formation, those observed usually fell soon after the segments had been laid down. As noted

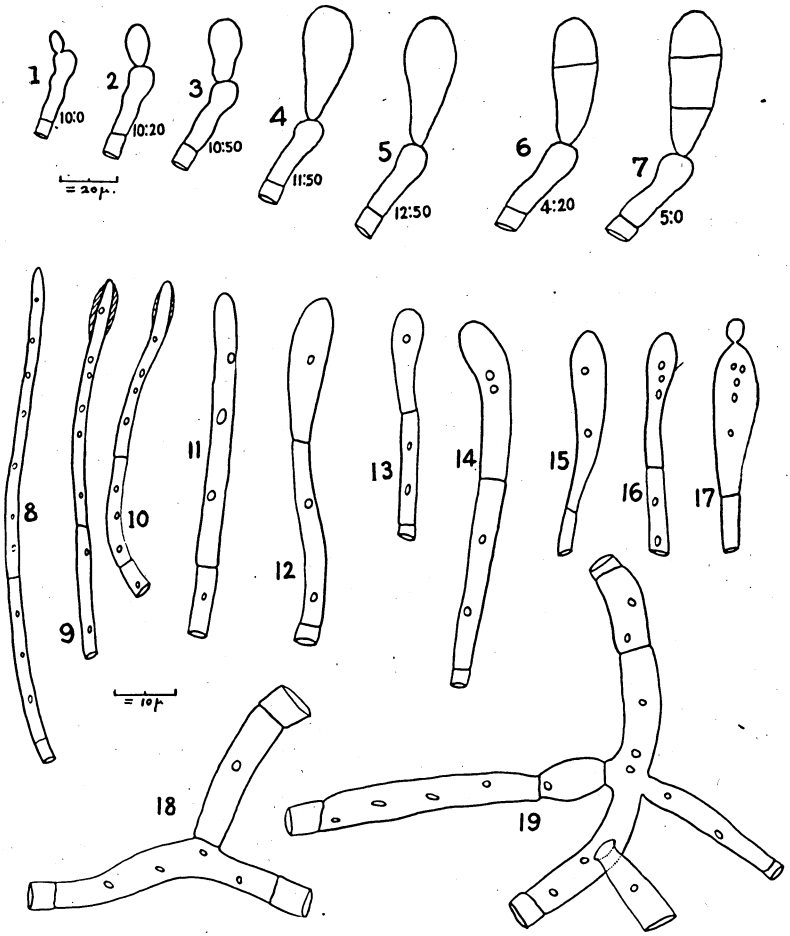


Fig. I. *Helminthosporium pedicellatum*

Drawings 1-7 were made with the aid of a camera lucida and a Leitz microscope using a 1/6th objective, a 10x eyepiece, and a tube length of 190 mm. The remaining drawings were made with a 1/12th oil immersion lens, a 12x eyepiece, and a tube length of 170 mm.

1-7. The course and rate of development of the conidium from its inception until segmentation started.

8-11. The tips of vegetative hyphae showing their form and nuclear content. Nos. 9 and 10 show the mucilaginous sheath.

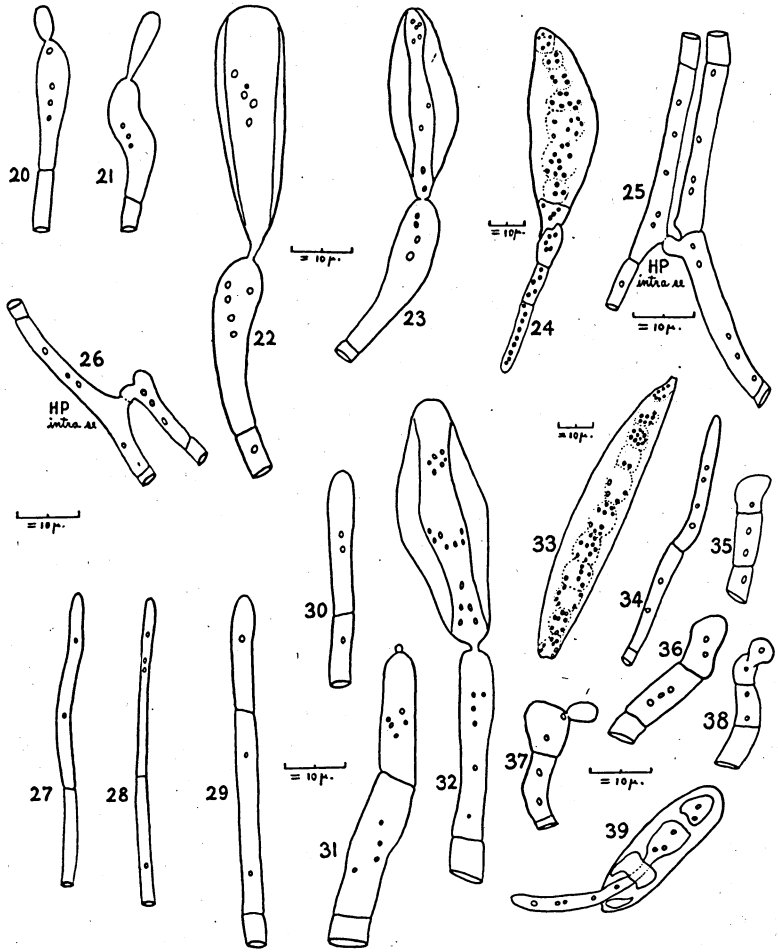
12-17. The conidiophore development and nucleation.

18-19. Hyphae from the older part of a mycelium showing the nuclear content.

Fig. II. Helminthosporium spp. (See opposite page.)

All drawings except Nos. 24 and 33 were made with the aid of a camera lucida, using a Leitz microscope and a 1/12th oil immersion objective, a 12x eyepiece, and a tube length of 170 mm. Nos. 24 and 33 were made when using a 1/6th objective, a 12x eyepiece and a tube length of 170 mm.

20-23. Stages in the conidial formation of *H. pedicellatum.* Note the characteristic conidiophore shape, and the endosporium formation in 22-23.



24. A germinating conidium of *H. pedicellatum.* The number and position of all the nuclei were determined under an oil immersion objective. The conidial segment edges are hazy in the original, and so are only dotted in the drawing.

25-26. Two hyphal fusions from the older part of the mycelium in *H. pedicellatum.* Notice the equal distribution of nuclei in the halves of the fusion cells, and the formation of a hypha in the cell just above the right half of the fusion cell.

27-28. The tips of vegetative hyphae of *H. monoceros* (compare Nos. 29, 30). These tips show the smaller nuclear content characteristic of less active hyphae.

29-32. The conidiophore form and nucleation, the conidial inception, and approach to maturity in *H. monoceros.*

33. A mature conidium of *H. monoceros,* showing the nuclear content of the segments. The number and position of all nuclei were determined under an oil immersion lens. The segment outlines, being hazy in the original, are only dotted in the drawing.

34. The tip of a vegetative hypha in *Helminthosporium sp.* (BN). Notice the form and nuclear content.

35-38. The conidial inception, the nuclear content, and the form of the conidiophore in *Helminthosporium sp.* (BN).

39. A germinating conidium of *Helminthosporium sp.* (BN). Notice that the median segment has germinated and pushed past but not fused with both the penultimate and terminate segments on its way to the exterior through the base of the conidium.

by previous authors, the conidia on falling leave an obvious scar, and the geniculation of the conidiophore usually is characteristic. The conidiophore cells, from which later conidia were formed, did not invariably contain one nucleus.

Brierley (5) has referred to the "multicellular spores with, as a rule, uninucleate cells such as Fusarium spp., Helminthosporium spp., etc." In the three species of Helminthosporium used, the small, sometimes deformed, and often rather transparent conidia might contain only one nucleus in each segment. The normal conidia, when mature, had from three to six, or even more, nuclei in each segment (Fig. II. 24, 33, 39), but the terminal segments frequently had a lower number than the median ones.

On the germination of the conidia in the species used, germ tubes were produced only from their ends, and in *H. pedicellatum* only as a rule from the basal end (Fig. II. 24). This, as Stevens (46) and others have indicated, is a specific character. If the terminal segment did not germinate, the next segment did, or the next, etc. An inner segment, however, invariably sent its germ tube out at the end of the conidium, and in doing so pushed past the intervening segments, and did not fuse with them (Fig. II. 39). Almost invariably the germ tube from an inner segment started from the dot-like thickened center of the transverse septum. In a few cases more than one segment had formed a germ tube, consequently the conidia had more than one germ tube, as Drechsler (24) found. When a conidium was cut in pieces with the microscissors (21), the uninjured median segments always germinated, even when germination of the terminal segments had already occurred before the conidium was cut.

Stevens (46) and others (15, 24) have stated that anastamoses of the germ tubes in their species were "not uncommon." In this investigation it was noted that while hyphal fusions were more common between the germ tubes of *Helminthosporium sp.* (BN), than in the other species, hyphal fusions were frequent in the mature mycelium of all three species (Figs. II. 25, 26; VI. 93-96). In the cytological preparations no unusual distribution of nuclei has been seen as would indicate migration of nuclei from one cell to the other. On the other hand, a few examples were found in which there was a nucleus in the anastamosis tube; and migration clearly had occurred to some extent in these cases.

Formation of Conidia and Nuclear Content in Fusarium spp.

In the two species of Fusarium, all the strains, their saltants, and cultures arising from isolated fusion cells have been examined, in both the living state and as cytological preparations. The mycelium was con-

siderably finer than in the species of Helminthosporium, and was made up of hyphae whose cells were invariably uninucleate (Fig. III). The only exceptions to this rule were certain stages in the formation of a conidium (Fig. III. 53, 54, 56), or before a transverse septum had been laid down after a nuclear division, or in cells formed by the fusion of two cells. This type of cell is referred to as a "fusion cell," and its behavior is described later.

The development of the conidia is of interest. They may be formed either on short side branches or conidiophores, or at the ends of hyphae (Fig. III. 40, 47, 48-61). In the latter case there appeared to be little or no differentiation into the somewhat pear-shaped organ, as in the case of the short side branches. At the end of the young conidiophore invariably in the strain $FB_{1.1}$, and frequently in the other strains, a cap of material was stained intensely by Haidenhain's haematoxylin and the Feulgen "Nuclealreaktion" (Fig. III. 48). The young conidium pushed out from a pore in this cap, and, swelling rapidly, left this stained area as a necklace at its base (Fig. III. 50). When the conidium had elongated to about two-thirds of its length, the tip bent slightly to one side (Fig. III. 54). The conidiophore nucleus divided by mitosis when the conidium had nearly or quite completed its growth, and one of the daughter nuclei passed at once into the conidium (Fig. III. 53, 54). Frequently at this time, but sometimes later, the protoplasmic connection between conidium and conidiophore was broken (Fig. III. 60). The conidium nucleus divided and a transverse septum was laid down (Fig. III. 57), the first usually being a median septum. The base of the conidium now showed a slight growth on the same side as the bend near the apex of the conidium (Fig. III. 58-60). In this way the familiar sickle shape of the mature conidium was achieved. In the meantime further septation of the conidium continued. While the conidia usually remained attached to the conidiophores until maturity, conidia with only one or no septum often have been seen, or have been broken away from their conidiophores. Such a conidiophore is shown in Figure III. 61. Later these conidia were seen to form septa. The time required for the formation of a conidium of $FB_{1.1}$ on Brown's medium (9) at 17° C. was about 4 hours (Fig. III. 40-47).

On germination of a conidium, the more usual manner was for one or both of the terminal segments to grow out and produce a hypha (Fig. III. 63). Quite often one or more of the median segments produced germ tubes; but in no case was any fusion between the segments of conidium ever observed (compare Zeller, 48); and only once was fusion between two segments of adjacent conidia noticed.

In the series of drawings of hyphal fusions, the migration of one of the nuclei into the opposing half of the fusion cell is shown (Fig. VI.

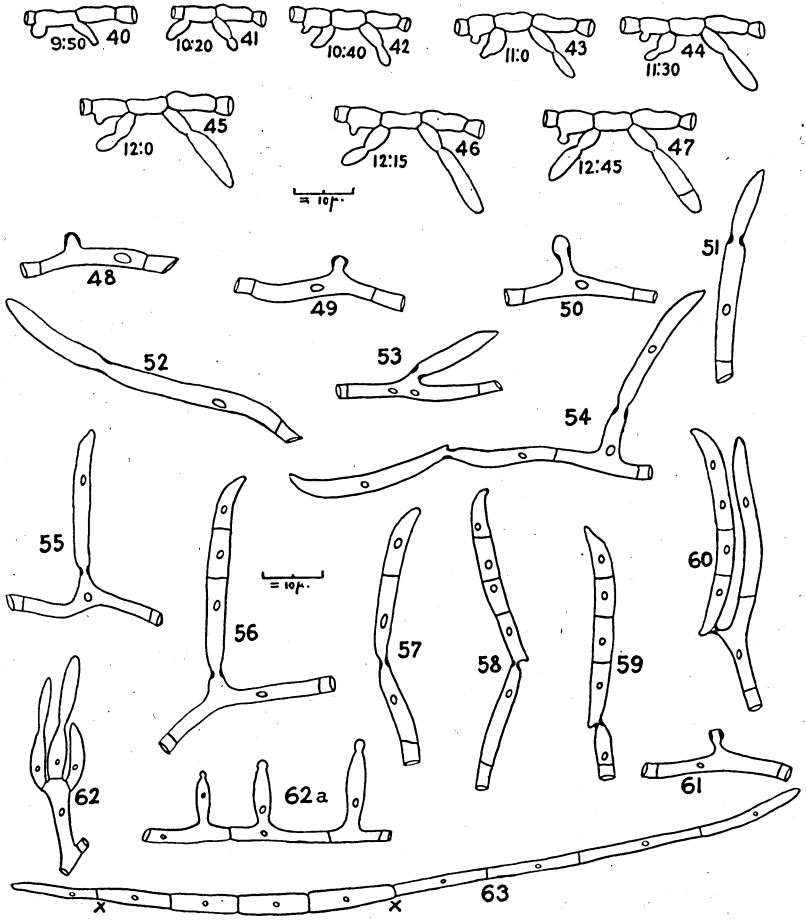


Fig. III. Fusarium spp.

All drawings were made with the aid of a camera lucida using a Leitz microscope and a 1/12th oil immersion objective, a 12x eyepiece, and a tube length of 170 mm.

40-47. The course and rate of conidial development in $FB_{1.1}$.

48-61. The formation and nuclear content of the conidium in $FB_{1.1}$. Notice the cap of deeply stained material on the conidiophore and the development of the base of the conidium. The conidium from the conidiophore shown in No. 61 had been broken off prematurely, hence the unusual form (compare with Nos. 48-49).

62, 62a. The beginning of conidial formation in FA_2.

63. A germinating conidium of *F. vasinfectum*. The x's mark the limits of the conidium proper.

88-92). In addition, the characteristic "kink" which can usually be seen in fusion cells is clearly shown. While later stages of fusion cells have been stained, and some cytological observations made on the development of isolated fusion cells, the evidence is not sufficient to justify comment. It can be stated, however, that no case of a fusion cell has been seen with less than two nuclei, and therefore there is no evidence of a nuclear fusion having taken place. Further observations on the formation and the development of fusion cells is given in Section II.

Cultural Experiments

An evaluation of the evidence given in the previous part is postponed until later, but it is clear that there is a uninucleate stage in some, if not all, the organisms used. This observation enabled a test to be made of the occurrence of heterocaryosis. For if a condition of heterocaryosis was present it would seem that cultures arising either from single conidia of Helminthosporium or from single conidia or cells of Fusarium would not all be the same. In other words, it was important to determine whether single conidia or cells, when isolated, grew into colonies similar to those of their parent strains.

Further, it was necessary to find out whether a change occurred in the colonies developed from the fusion cells of Helminthosporium spp. and the binucleate fusion cells of Fusarium spp.; for such cells would appear to be a possible starting point of a heterocaryotic condition. In addition, morphologically peculiar cells were isolated, for they might well give rise to colonies different from those of the parent strains.

During such experiments the occurrence and frequency of any changes were noted. Also the nature of such changes, whether saltation or not, was ascertained. Finally, at the end of the experiments, the strains, which had been thus treated in previous subculture generations, were tested under suitable conditions for their ability to saltate.

Methods

Unless otherwise stated, the medium consistently employed during the course of this investigation was Brown's synthetic potato agar (9), the H-ion concentration of which was adjusted when necessary to pH 6.8. Usually little or no adjustment was necessary. The stock cultures have been kept in test tubes on the same medium, and have been subcultured every two to three weeks, while about every two months single spore cultures have been made to insure genetic purity.

In the determination of the cultural characters, 250-cc. Erlenmeyer flasks containing 35 cc. of medium were used, and the parent strains were always tested along with derivative strains. In the light of

previous experience (20, 21) apart from that of other workers (8, 9), it seems to be impossible to write a sufficiently accurate description of the cultural characters of a strain that will enable one to avoid such comparison tests. Consequently, altho a table of the cultural characters of the strains and the saltants used is given (Table 2), it has not been used in the determination of the cultural characters. From this table it will be seen that the differences between the saltant strains and their parents is sufficiently great to enable the determination to be easily made, provided the cultures themselves are examined.

The single cells from a hypha, or single segments of a conidium were obtained by the microscissors method (22).[3] The procedure consisted in placing a suitable inoculum on an agar drop on a cover slip. The required single cells from the conidia or hyphae of this inoculum or from its subsequent mycelium were then separated by cutting the hypha or conidium in two on either side of the required cell with the microscissors. Such single hyphal cells, or conidial segments, were then isolated by carrying them, using the Dickinson method, to a fresh part of the agar. The blocks, or squares, of agar containing these isolated cells were next transferred with a sterile scalpel to test tubes containing the stock medium.

Observations

Turning now to the isolation experiments, in Table 3 there is given a list of the isolations that have been made, and that have all been tested alongside the parent strains for identification. From that table it will be realized that isolations were made of single conidia and single cells of various kinds. In addition, from the cultures arising from such isolations, single conidia and single cells were again isolated. This process of successive isolation of single conidia and cells was repeated a number of times for different types of conidia and cells. In every single isolation thus made, the resultant colony was the same as that of the parent. No change has ever been observed in any of these isolations.

[3] The microscissors was devised during this investigation. It consists of two upright triangular blades attached to movable arms by a rack and pinion. One arm is stationary and the other is movable along one plane. When the blades are moved, the tips come into contact and cut the hyphae with a scissors-like motion. The hyphae to be cut are grown on agar drops on cover slips, and the operation of cutting them is done under the microscope. The scissor blades are raised until the hypha lies between the two tips, the movable blade is then moved past the stationary one, thus cutting the hypha.

Table 2

Cultural Characters of the Strains and Saltants of Helminthosporium and Fusarium

Organism	Brown's medium				Richard's medium			
	Color	Luster	Surface and topography	Edge	Color	Luster	Surface and topography	Margin
H. pedicellatum HP	Mummy brown	Dull	Slightly radiate	Notched	Sooty black	Dull, powdery in center	Few hyphal tufts, radial depressions in center	Entire
H. monoceros HM	Chestnut brown	Dull	Radiate	Notched	Aniline black	Dull, powdery	No depressions or hyphal tufts	Entire
H. species BN	Mars brown	Dull	Zonate	Entire	Bone brown	Dull	Slightly zonate	Entire
H. species BW	White	Dull	Smooth	Entire	Drab gray	Dull, slightly powdery	Spotted more or less with hyphal tufts	Entire
F. fructigenum FA_2	White	Felted, later dull	Fluffy at first; later flat	Fimbriate	Pale salmon	Felted	Zonate with a few radial depressions	Fimbriate
F. fructigenum $FB_{1.1}$	Light salmon orange	Waxy, somewhat polished	Very finely verrucose	Fimbriate	Grenadine	Somewhat polished, waxy	Verrucose	Fimbriate
F. fructigenum $FB_{1.1\text{-}1}$	White	Felted, later dull	Fluffy at first; later flat	Fimbriate	Light ochraceous salmon	Dull	Slight mycelial felt. Verrucose	Fimbriate
F. fructigenum $FB_{1.1\text{-}2}$	White	Waxy, somewhat polished	Very finely verrucose	Fimbriate	White	Somewhat polished, waxy	Verrucose	Fimbriate
F. vasinfectum var., *lutulatum* FV	Seashell pink	Waxy, polished	Finely zonate radially striate	Entire	Flesh ocher	Polished, waxy	Numerous radial "lines" and upright conical processes ⅛–¾ in. high in center	Entire

The colors for this table were determined by comparison with Ridgway's Color Standards and Color Nomenclature.

Table 3

Number and Type of Successful Isolations Made from Inoculum Grown on Brown's Medium

Type of isolation made	*H. pedicellatum* HP	*H. monoceros* HM	*H. sp.* BN	*H. sp.* BW	*F. fructigenum* FA_2	*F. fructigenum* $FB_{1.1}$	*F. fructigenum* $FB_{1.1-1}$	*F. fructigenum* $FB_{1.1-2}$	*F. vasinfectum* FV
Number of single conidial isolations .....(large)	27	29							
(medium)	19	29							
(small)	11	18	46	—	36	33	18	5	20
Number of successive single conidial isolations	5	5	4	—	6	6	3	—	3
Number of single hyphal cell isolations	16	50	41	7	16	12	20	—	17
Number of successive single hyphal cell isolations.....	1	4	3	—	2	2	—	—	—
Number of single conidial segment isolations									
(terminal segment)	3	—	—	—	7	12	10	—	10
(median segment)	2	—	—	—	4	5	6	—	7
Number of successive single conidial segment isolations..	1	—	—	—	3	3	2	—	—
Number of fusion cells isolated (intra se)	10	8	7	—	7	8	4	—	—
Number of morphologically peculiar single cells isolated...	3	18	15	—	—	—	—	—	—
Total of successive single conidial or cell isolations....	7	9	7	—	11	11	5	—	3
Total of single conidial or cell isolations	91	143	109	7	70	70	58	5	54

It should be realized that these isolation experiments were made as an adjunct to the cytological observations, and that the value of the deductions made from them depends on the appreciation of this fact. Further, it should be realized that no attempt was made to "educate" the strains by repeated isolations of a particular type. Consequently, while these observations are in agreement with those of Mohendra (37), they differ in that he compared the growth in successive subculture generations using three different sources of inoculum, namely, young mycelium, old mycelium, and spores.

During the course of this work only two saltations have occurred on Brown's medium. In one case the saltating culture was an old stock culture of *Fusarium fructigenum* $FB_{1.1}$, which through an oversight had not been discarded when it was subcultured. This saltant was numbered $FB_{1.1-1}$. The other case was an almost sterile form of *Helminthosporium sp.* which appeared only once in a flask culture; this strain was called

BW in distinction to the parent strain BN. In various strains of *Helminthosporium sativum* and other species a number of probable saltations were observed on Brown's medium. These strains were in consequence not used in the investigation.

Having thus established the purity and comparative stability of the strains on Brown's medium, it was necessary to test their ability to saltate. While Christensen (16) had shown that high temperatures were conducive to saltation in Helminthosporium spp., Brown (10) had found that a rich medium, Richard's, had the same effect in Fusarium spp. As it was only desired to know that saltations could be produced, the easier method appeared to be that of growing the strains on Richard's medium. In the cultures produced on this medium a number of sectors appeared, and out of these six were chosen for further study (see Table 4). In each of the six cases it was found that the cultures arising from these sectors were distinct in cultural characters from the parent strains. Single conidial and single cell isolations were made and the distinctions still persisted. Furthermore, these six strains have remained constant in cultural characters over at least eight subculture generations. Consequently, it was considered that the culture arising from these sectors had behaved as saltations from, and not as environmental variations of, the parent strains. From these observations it was clear that the single cell isolations, which had been made in the previous subculture generations of the parent strains, had not eliminated the capacity of these parent strains to form sectors, or to saltate.

Table 4

Saltation Observed in Richard's Medium

Organism	Strain designation	No. of previous successive single conidial or cell subculture generations	No. of types of saltants determined by isolation	No. of sectors observed (approx.)
H. pedicellatum	HP	6	0	2
H. monoceros	HM	6	0	12→
Helminthosporium sp.	BN	5	2	5→
F. fructigenum	FA_2	5	1	8→
F. fructigenum	$FB_{1.1}$	5	3	40→
F. vasinfectum	FV	2	0	0→

Discussion

At this stage it seems best to evaluate the observations and experiments described. The foregoing evidence brings out three points, namely, the uninucleate stage or condition; the failure to get saltation by single cell isolation on Brown's medium; and, lastly, the ability to form saltations on an appropriate medium.

While the uninucleate condition of the segments of the conidia and the mycelium in the Fusarium strains is unquestionable, there may be some query as to the condition in the young conidiophore of the Helminthosporium types. The recognition of the young conidiophore is important and is based upon its shape, and in *H. pedicellatum* this was striking enough for Henry to describe as clavate (31). Drechsler (24) has remarked on the brown tint in the mature conidiophore wall, other than at its tip, in *H. monoceros*. By their direction of growth perpendicular to the medium a large number of hyphae can be eliminated. In consequence, it is considered that by using these three criteria the young conidiophores can be reliably recognized, except perhaps in *H. monoceros*. The nuclear condition of the apical cell in such identified conidiophores is less open to discussion. The shorter ones have invariably one nucleus, the longer ones about three. The intermediate ones contain from one to two, the product of mitosis. In contrast, the vegetative hyphal number is normally between four and six.

It may be said that this nuclear condition is a response to the conditions under which the culture was grown, namely, in a liquid medium on a glass slide. This is not considered probable in view of the fact that the conidial form and the mycelial development is normal, and further because the same result was obtained when the concentration and the relative proportions of the carbohydrate and the nitrogen sources were varied.

In the case of the isolation experiments, failure to get any change except in the two cases described is of questionable value for evidence. Judged alone, the number is not considered sufficient to justify any conclusions. However, when it is known, for example, that on 62 different occasions *H. pedicellatum,* HP, and on 63 different occasions *F. fructigenum,* FA_2, have been grown apparently from a single nucleus, and that on each occasion the resulting colony was the same as the parent colony, then the replication appears to be ample. Consequently it is thought that the combination of cytological observations and isolation experiments provide conclusive evidence that all the nuclei in each strain are of the same type—in other words, that these strains are homocaryotic.

The logical step following this conclusion was to see if the strains that had been so examined and treated could or would at this stage produce new forms. The experiment was made with Richard's medium only. Three of the strains saltated, or, in other words, formed sectors or islands of abnormal appearance in their colonies. Isolations from such areas gave colonies different from the original parent, and further subculturing did not change their appearance.

As some of these strains have been shown to be homocaryotic, and have the ability to saltate, it is clear that the nature of this saltation is not one that is due to the separation of different kinds of nuclei, associated together in the same cell. It is also equally clear that, in these fungal strains, hyphal fusions have not served as a mechanism for an association and dissociation of nuclei of different kinds.

EXPERIMENTAL SECTION II

CYTOPLASMIC INHERITANCE

Formation of Hyphal Fusions

Almost any piece of the older part of a mycelium, when examined under a microscope, will show some apparently normal hyphal fusions. When, however, such fusions are examined with an oil immersion objective many of them will be found to be not real but apparent or attempted fusions. The real fusions can be verified by observing the passage of granules from one component of the "fusion cell" to the other. The attempted fusions differ in usually having intact a double wall between the component cells.

In addition, two types of fusion cell can be distinguished. In the first, the fusion cell is produced by the tip of a normal hypha growing up to another hypha, lying at right angles to it or at an angle to it, and, on reaching the opposing hypha, fusing with it (Fig. IV. 64, 69). The other sort is formed between two hyphae lying nearly or quite parallel. In such cases the resultant fusion cell is H-shaped, the pair of cells lying side by side having fused by the formation of a bridge between them (Fig. IV. 70). In both these types real and attempted fusions have been found, and it is considered that their difference is only one of morphology.

The formation of both real and attempted fusions is illustrated by series of camera lucida drawings made at suitable intervals (Fig. IV. 64-69, 71-73). These drawings show that in the real fusions both the participating cells play an active part, while in the attempted fusions only one cell, as a rule, takes part.

When one hypha is growing toward another hypha and is about to form a real fusion, the opposing hypha, when the oncoming hyphal tip is about twice its own diameter away (Fig. IV. 65), puts out a short hypha; then the actual fusion occurs between the two hyphal tips (Fig. IV. 66-68). Sometimes more than one short hypha is put out by the opposing hyphae (Fig. IV. 65).[4] Almost always these hyphal tips do not meet end on, but slightly to one side thus forming a kink at the

[4] I am indebted to Professor A. H. R. Buller for drawing my attention to this phenomenon.

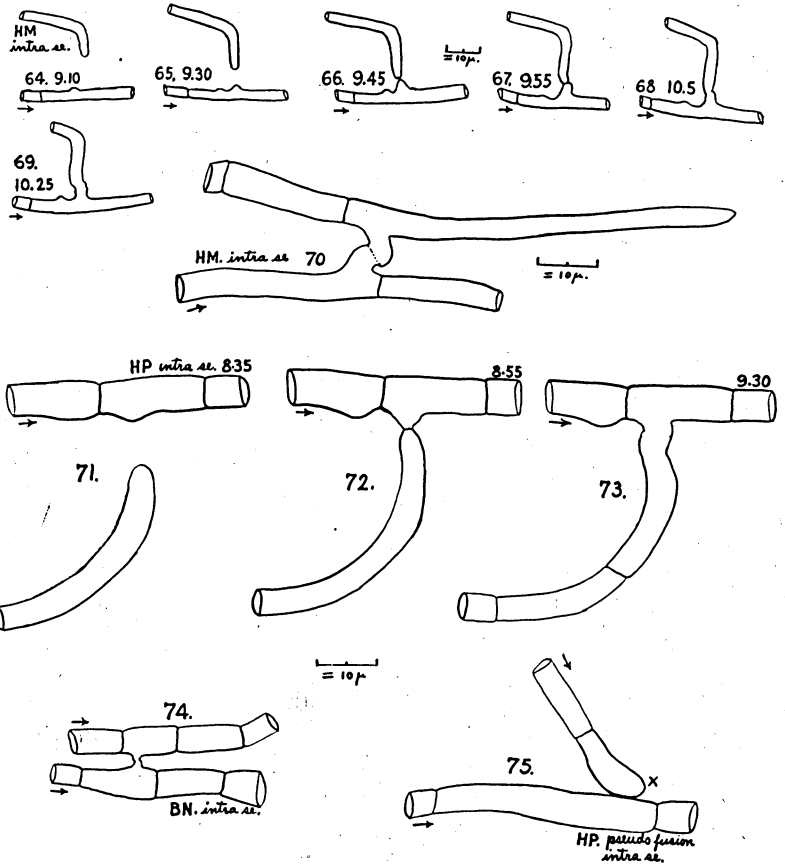


Fig. IV. Helminthosporium spp.

All drawings except Nos. 64-69 were made with the aid of a camera lucida using a Leitz microscope and a 1/12 oil immersion objective, a 12x eyepiece, and a tube length of 170 mm. Nos. 64-69 were made using a 1/6th objective, a 12x eyepiece, and a tube length of 170 mm.

64-69. The formation of a fusion cell in *H. monoceros.* Notice in No. 65 that an attempt is being made to form a second hypha for the fusion, and that this second hypha is successful (No. 68). The asymmetrical meeting of the two hyphae (67), and the persistence of the resulting "kink" (69) are characteristic.

70. A fusion cell from the older part of a mycelium of *H. monoceros.*

71-73. The formation of a fusion cell in *H. pedicellatum.* Notice again that the fusion cell is formed by both participants.

74. An H-shaped fusion cell formed between two hyphae lying parallel in *Helminthosporium sp.* (BN.) Notice again the characteristic "kink."

75. An example of an attempted fusion in *H. pedicellatum.* The upper hypha has grown down to the other hypha without evoking any response. It has pressed itself up against the other hypha without result, and is now about to continue its growth at x.

point of contact (Fig. IV. 67). This is probably due to circumnutation of the growing tips. When H-shaped fusion cells are formed, the process is similar in all respects; hence it appears that some stimulus is transmitted from, or induced by, the oncoming hypha in the opposing hypha. This suggestion is supported by the following observations on attempted fusions.

On the other hand, when an attempted fusion is formed, only the one hypha usually takes part, and grows right up to the other hypha and presses against it, as tho forming an appressorium (Fig. V. 80, 82, 86). As a rule no breaking down of the wall occurs in such cases. Sometimes the opposing hypha has been seen to put out a short hypha. Usually after a short time the active hypha continues its growth from one side and passes over or under the passive or opposing hypha. The appressorium-like bulge is left behind as evidence of the attempt at fusion (Figs. IV. 75; V. 86).

One example deserving particular mention was a real fusion observed between a growing hypha of *H. pedicellatum,* which came up to an opposing hypha of *H. monoceros* (Fig. V. 85). The walls between the two cells were broken down, as could be seen by careful focussing with an oil immersion objective. No granules were observed to pass from one component to the other, and very shortly after the fusion was complete the contents of the *H. pedicellatum* cell were seen to become distinctly granular. No further change had taken place after 48 hours, and no growth resulted after isolation.

During the course of these experiments, fusions or attempted fusions have been observed between almost all the strains used. In Table 5 is shown the number and the kinds of fusion observed. From this table it will be seen that, while no real fusions have been observed, with one possible exception, between strains of the Helminthosporium and Fusarium types, some possible real fusions have been observed between the species of Helminthosporium (Fig. V. 83, 85). Real fusions, however, have been seen frequently between the two species in Fusarium (Fig. V. 79), and between saltants and their parent strains (Fig. V. 87).

Sometimes more than one attempt at fusion may be made between two cells, especially when a growing hypha is approaching another hypha (Figs. IV. 64-69; V. 82). However, no case has as yet been seen of two successful fusions between two cells, but several times one cell has been found to form more than one successful fusion with other cells (Fig. V. 84).

No example of growth from a fusion cell has as yet been found, except when the participating hyphae have been isolated. Usually, growth from a fusion cell only takes place when the cell itself has been isolated or when it is adjacent to a killed cell. Such observations show,

however, that fusion cells are capable of growth. It will be recalled that it was shown in Section I that fusion cells produced colonies similar to the parent colonies.

Table 5

Number and Kind of Hyphal Fusions Observed Between Certain of the Organisms Used*

Organism	HP	HM	BN	BW	FA_2	$FB_{1.1}$	$FB_{1.1\text{-}1}$	$FB_{1.1\text{-}2}$	FV
H. pedicellatum									
Perfect fusion ..	+++								
Attempted fusion	++								
H. monoceros									
Perfect fusion ..	?++	+++							
Attempted fusion	++	?0							
H. sp., BN									
Perfect fusion ..	1?++	?1	+++						
Attempted fusion	++	++	++						
H. sp., BW									
Perfect fusion ..			+++	+++					
Attempted fusion			?0	++					
F. fructigenum FA_2									
Perfect fusion ..	0		0		+++				
Attempted fusion	++		++		++				
F. fructigenum $FB_{1.1}$									
Perfect fusion ..	?++	0	0	0	+	+++			
Attempted fusion	++	++	++	++	++	++			
F. fructigenum $FB_{1.1\text{-}1}$									
Perfect fusion ..					0	+++	+++		
Attempted fusion					++	++	++		
F. fructigenum $FB_{1.1\text{-}2}$									
Perfect fusion ..						+++		+++	
Attempted fusion						++		++	
F. vasinfectum									
Perfect fusion ..	0		0		0	++	0		+++
Attempted fusion	++		++		++	++	++		++

* +++ = numerous; ++ = some; + = few; 0 = none.

Production of Hyphal Fusions

For the purpose of isolating the product of fusion between two cells or segments from hyphae of known origin, it was necessary to have such a fusion taking place on the surface of an agar drop on a cover slip. However, hyphal fusions were of such common occurrence that at first it seemed as if all that was necessary to make one germ tube fuse with another was to allow two conidia to germinate in close proximity. It was soon found, however, that under such conditions the germ tubes would rarely fuse, only one case of fusion having occurred

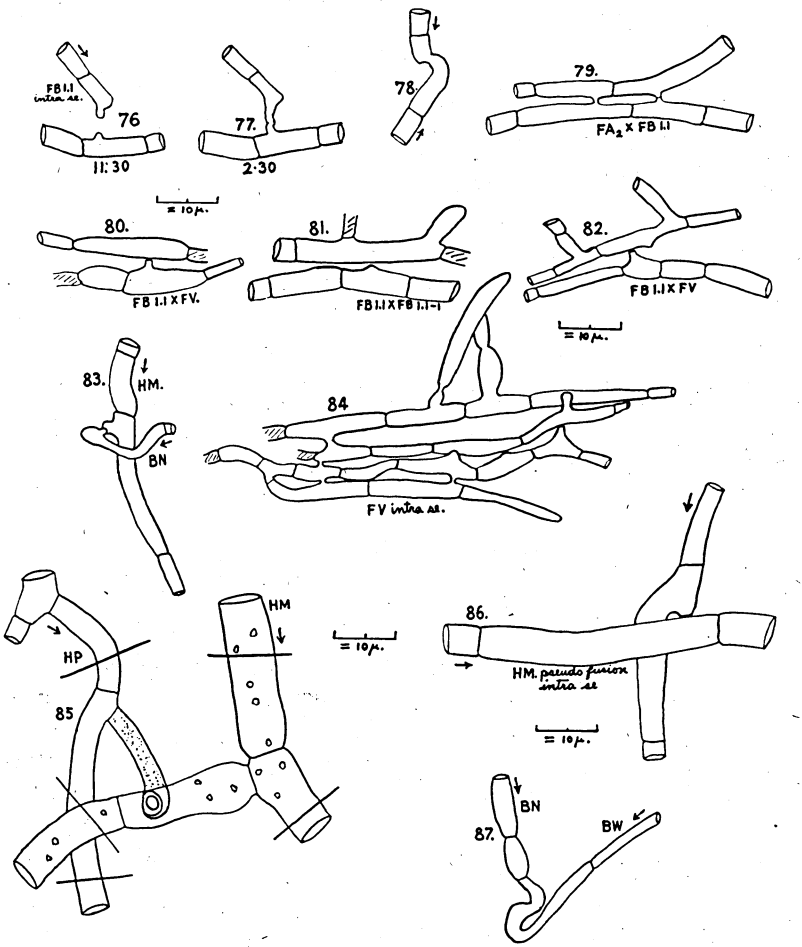


Fig. V. Hyphal fusions

All drawings were made with the aid of a camera lucida using a Leitz microscope and a 1/12th oil immersion objective, a 12x eyepiece, and a tube length of 170 mm.

76-77. The active participation of both cells in the formation of a fusion cell in $FB_{1.1}$.

78. A fusion cell in $FB_{1.1}$ showing the characteristic "kink."

79-82. Real and attempted fusions in Fusarium spp. Notice in the pseudo-fusions of Nos. 80, 82 the flattened tip of the active hypha, which was pressed against the opposing passive hypha. For demonstration purposes the active hypha has been moved slightly away from the other hypha.

83. A fusion (?real) between *H. monoceros* and *Helminthosporium sp.* (BN). Notice how the BN hypha has grown round the HM hypha and that both participants are active.

84. Fusions in hyphae of *F. vasinfectum* lying side by side. To the left there are four cells fused together, one with the other.

85. A fusion (?real) between *H. pedicellatum* and *H. monoceros*. The black lines mark the cuts made in isolating the fusion cell. See the text for description.

86. An attempted fusion in *H. monoceros*. The active hypha had grown up to the opposing hypha without evoking a response. After pressing futilely against the opposing hypha, it had continued its growth from one side.

87. A real fusion between *H. sp.* (BN) and its saltant *H. sp.* (BW) showing the characteristic "kink."

in some 500 pairs of Fusarium conidia placed together. On the other hand, in the case of the germ tubes of *Helminthosporium sp.*, if the conidia were placed together in pairs under such conditions, they fused rather more frequently. It was therefore necessary to find some environmental factor that would stimulate fusion, presupposing that fusion was not an entirely haphazard phenomenon.

Starting with the observation made by Brodie (7), mentioned previously, that fusions between the germ tubes of his Coprinus conidia tended to be more frequent under crowded conditions, it was soon found that this was apparently the case with all the organisms used. It is as well to repeat "apparently," as the frequency of hyphal fusions was judged by observation rather than by counting and also because the frequency per unit number of conidia germinating in a given area was not ascertained. In any case fusions produced under such conditions were not suitable for isolation owing to the difficulty of determining the origin of the component hyphae, and because such fusions, usually, were not produced until about twenty-four or more hours after germination.

A comparison also was made between the occurrence under the same conditions when hyphae formed the inoculum. Here the observation was made that fusions occurred only in developing colonies some distance behind the growing edge of the colony; consequently again such fusions were not suitable for isolation. Both Matsumoto (36) and Ezekiel (25) observed that the hyphae of meeting colonies in Rhizoctonia and Sclerotinia fused with each other, but in the strains of Helminthosporium and Fusarium used in this investigation this did not occur.

As fusions were seen only in the older parts of a mycelium, it seemed that staling substances might stimulate their production. Therefore a sterile staled liquid was prepared by filtration, mixed in varying quantities with cool liquid agar, and pairs of conidia were allowed to germinate on it. No more fusions were obtained than in the control preparations.

In an article on graminicolous Helminthosporia, Drechsler (24) has described the occurrence of numerous fusions occurring when hyphae were growing next to a glass surface. Consequently agar drops with spore inoculum were inverted on cover slips and the germination was watched. Such conditions caused the production of hyphal fusions to a considerable extent, tho not until about twenty-four hours after germination. As fusions are also produced not in contact with glass, it did not seem that a contact stimulus was the causative agent; instead, it appeared more probable that some volatile metabolic product tended to stimulate fusions.

Following such a suggestion and after obtaining a negative result with a bicarbonate (c.f. Pratt, 40), the effect of growth in high concentrations of CO_2 and N_2 was tested, but again no increase in the number of fusions resulted. On the other hand, it was found that the presence of Fusarium conidia germinating in the neighborhood of *H. pedicellatum* conidia stimulated the production of fusions between the hyphae of the latter.

From the experiments recorded above, the results of which are given in Table 6, it was concluded that possibly the production of fusions tended to be stimulated by some metabolic substance that was volatile or easily altered.

Table 6

The Frequency of Hyphal Fusion* under Different Cultural Conditions in the Mycelium of Helminthosporium pedicellatum 48 Hours after Germination of the Conidia (or Mycelium) with a Temperature Ranging Between 17° and 22° C.

Cultural conditions	Position of inoculum		
	Conidia on top of agar	Conidia on agar next to cover slip	Conidia on agar between 2 cover slips
Control 1.5% agar	2	3	1
1.5% agar + N/2 stale liquid	1	2	0
1.5% agar + N/10 stale liquid	2	3	0
1.5% agar in atmosphere CO_2	1	2	—
1.5% agar in atmosphere N_2	1	2	—
1.5% agar in CO_2 + N/2 stale liquid	1	—	—
1.5% agar in N_2 stale liquid	1	2	—
1.5% agar + 5% cane sugar	3	4	2
1.5% agar + 2% dextrose	3	4	2
1.5% agar + 1% KNO_3	3	3	2
1.5% agar + 2.5% cane sugar + N/2 stale liquid	3	4	2
1.5% agar + 2% asparagin	2	3	1
1.5% agar + 5% KH_2PO_4	2	3	1
1.5% agar + 2.5% $MgSO_4$	2	3	1
1.5% agar + 1% NH_4OH	2	3	1
Brown's medium (control)	3	4	1
Brown's medium (control) + Fusarium spp. $FB_{1.1}$ condia	4	5	2
	Mycelium on top of agar	Mycelium on agar next to cover slip	Mycelium on agar between 2 cover slips
Brown's medium	3	4	2

* Key—5 = very many; 4 = many; 3 = some; 2 = few; 1 = very few; 0 = none.

Previously it had been noticed that if an inoculating needle was drawn across the younger part of a culture numerous fusions were formed between the hyphae pushed together by the movement of the needle. From this observation it was but a step to take two hyphae out from a culture and lay them side by side on a clear part of the agar. Numerous

fusions were seen to develop between such hyphae. On repetition it was found that fusions tended to be produced more frequently close to where the hyphae had been cut in separating them from the parent mycelium. In Table 7 are given counts of the number of fusions which occurred less than 3 cells from a cut cell, and more than 5 cells away. It will be seen that nearly double the number occurred close to a dead cell.

Such a result might very well be produced by the physical shock occasioned in the transfer of the hyphae to the new part of the agar surface, or by the physical shock of an adjacent cell having been cut in half and killed. But as fusions are frequently produced without such a physical shock, it seemed more probable to consider that the causative agent was some product of metabolism which diffused out in considerable amount from a killed cell. This supported the suggestion previously made.

The next step was to cut out lengths of hyphae containing two cells each and lay them alongside each other. The result was that in most cases each cell fused with the opposing cell in the adjacent hypha. In other words, in each pair of hyphae two fusions had been formed. In Table 7 a number of counts of fusions so produced are listed, and it will be seen that in more than 75 per cent of the cases, two fusions had been formed in each pair of hyphae.

In addition, fusions were found to be produced as easily with the younger parts of the growing hyphae as with the older parts. On the other hand, it was discovered that if the hyphal pairs were not put together as soon as they were cut out, then few or no fusions resulted. This was verified by cutting hyphae out and leaving them for an hour and a half before putting them together. In such cases practically no fusions were produced. High temperatures, about 26° C., also were found to hinder the formation of fusions.

All these later observations can be interpreted by the suggestion that physical shock induces hyphal fusion, just as well as by the suggestion of the presence of a product of metabolism that promotes hyphal fusion. However, until the critical experiment of growing hyphae in fresh extract of mycelium has been made it seems best to leave the question open, with, however, the suggestion that, in view of the early experiments, it seems more probable that the presence of a product of metabolism is the causative agent. Such a suggestion is also supported by the observations made on fusion cell formation, when, it will be recalled, it appeared that a stimulus might be transmitted from an oncoming hypha to the opposing hypha, and in this way the latter was induced to produce a short hypha. The actual fusion took place between the two hyphal tips.

Table 7

Production of Hyphal Fusions in Fusarium fructigenum, $FB_{1,1} \times FB_{1,1-1}$

Type of experiment	Experiment numbers																Total	Summary for each type of experiment				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		Temp. about 17° C.	Temp. about 26° C.	Delayed pairing of cells	Pairs of young cells	Pairs of old cells
No. of fusions 3 or fewer cells from dead cell	27	20	45														92					
No. of fusions more than 5 cells from dead cell	12	11	18														41					
2 × no. of pairs of cells put together				16	28	6	42	36	38	28	18	32	24	28	36	14	336	94	52	94	46	60
No. of fusions obtained at about 17° C.				15			33	26									74	74				
No. of fusions obtained at about 26° C.					3	2					1						6		6			
No. of fusions obtained when cells put together 1½ hours after cutting									1	6				2			9			9		
No. of fusions obtained with cells near to growing point												25				12	37				37	
No. of fusions obtained with cells some distance from growing point													18		24		42					42

Procedure Adopted for Obtaining Fusion Cells

From the experiments just described, the following method of producing and isolating fusion cells was evolved. Colonies of the required strains were grown on either side of an agar drop (Brown's medium) on a cover slip. Such cover-slip cultures were kept, when not under the microscope, on cork strips in petri dishes lined with damp filter paper. From the hyphae of these mycelia, 2 cell lengths were cut out and placed in pairs midway between the two colonies. It was as easy to pair two 2-celled hyphae from the same colony as to take one from each colony. In this way the parentage of the paired hyphae could be ensured. The magnification used in this operation was approximately 1,350.

As the paired hyphae had usually produced fusions and a slight growth from the non-fusing cells in about four hours, they were normally left about six hours. The parent colonies were then cut away, leaving the center part of the agar drop. This agar piece was now inverted on a new sterile cover slip, and an examination was made through the cover slip, usually with an oil immersion objective. The magnification used in such a case was about 2,000. A camera lucida drawing was made of every fusion cell and, if any doubt of fusion was entertained, the criterion used was the passage of granules from one component cell to the other.

Fresh, cool, liquid agar was added to the inverted agar piece. This liquid agar never came in contact with the living cells, as these were at this time between the cover slip and the pre-existing agar drop. After gelation, the now much larger agar slab was placed, paired hyphae uppermost, on a new sterile cover slip. The fusion cells were cut out with the microscissors and transferred to the fresh part of the agar. Slabs of this fresh agar, carrying the now isolated fusion cells, were placed on new sterile cover slips and left to grow.

Sufficient growth normally had taken place in about twelve hours, and the agar blocks after that time were again inverted on new sterile cover slips for drawing and high-power observation. Following this observation, fresh, cool, liquid agar was added and the enlarged agar drop placed, fusion cells uppermost, on a new sterile cover slip. The hyphae produced from the fusion cells were then cut out, and each was transferred separately to a piece of the fresh agar and their location marked. Squares of the fresh agar, each bearing one hypha whose origin was known, were now transferred with a sterile scalpel to test tubes containing the stock medium.

The repeated transfers to sterile cover slips and the two transfers to fresh agar were to guard against contamination from the air and from the parent colonies during manipulation. The percentage contamination from the air was under 3 per cent, thus showing the effectiveness

of the procedure. As a safeguard against parent-colony contamination, no case of which was noted, the first signs of growth on the isolated agar squares in the test tubes were carefully watched. If any case had occurred in which growth had started from the side of the agar square instead of from the center of the upper surface—where the isolated hypha was situated—it would have been discarded. A further safeguard was provided by transferring control agar squares from the same agar drop. These uniformly produced no growth.

Later, single spore isolations and mass inoculations were made to determine the purity and cultural characters of the isolated hyphae, produced by the fusion cells. The results of such experiments follow.

Progeny of Fusion Cells

Following the procedure already described, fusions were induced between certain strains, and the progeny were studied. When making such experiments, the relative frequency with which fusion cells were induced was easily determined. When fusion cells were induced *intra se,* the percentage of the total possible number was always high (see Table 8). The total possible was calculated as twice the number of pairs of hyphae, and each containing two cells, placed together. Furthermore, this percentage was high where strain $FB_{1.1}$ and its saltant $FB_{1.1-1}$ were used. On the other hand, when different species or strains were used as the parent strains, viz., $FA_2 \times FB_{1.1}$, $FA_2 \times FB_{1.1-1}$, $FB_{1.1} \times FV$, and $FB_{1.1-1} \times FV$, the percentage was low or nil. These figures, even if they are considered small, show clearly that fusions were more easily induced between cells of the same strain, or between cells of closely related strains, than between cells with a more distant relationship. It was instructive, also, to find that, altho FA_2 was similar in cultural characters to $FB_{1.1-1}$, no fusion cells were obtained. It should be noted that these results can not be taken to indicate that fusion is not possible between FA_2 and $FB_{1.1-1}$, but only that fusion cells have not as yet been obtained.

In Table 9 are given the results from the isolations of growth from isolated fusion cells. The identity of the isolations was determined by comparison cultures as previously described. It is much to be regretted that the numbers are not larger. However, in spite of this drawback, it is clear that no new or intermediate forms have been obtained in any of the 20 isolated fusion cells, induced *inter se,* which were so treated. Furthermore, in the control experiments, when 19 fusion cells, induced *intra se,* were treated, no new forms were produced. This result is considered significant. In three cases mixtures of the parent strains were obtained, and the significance of this result is discussed later.

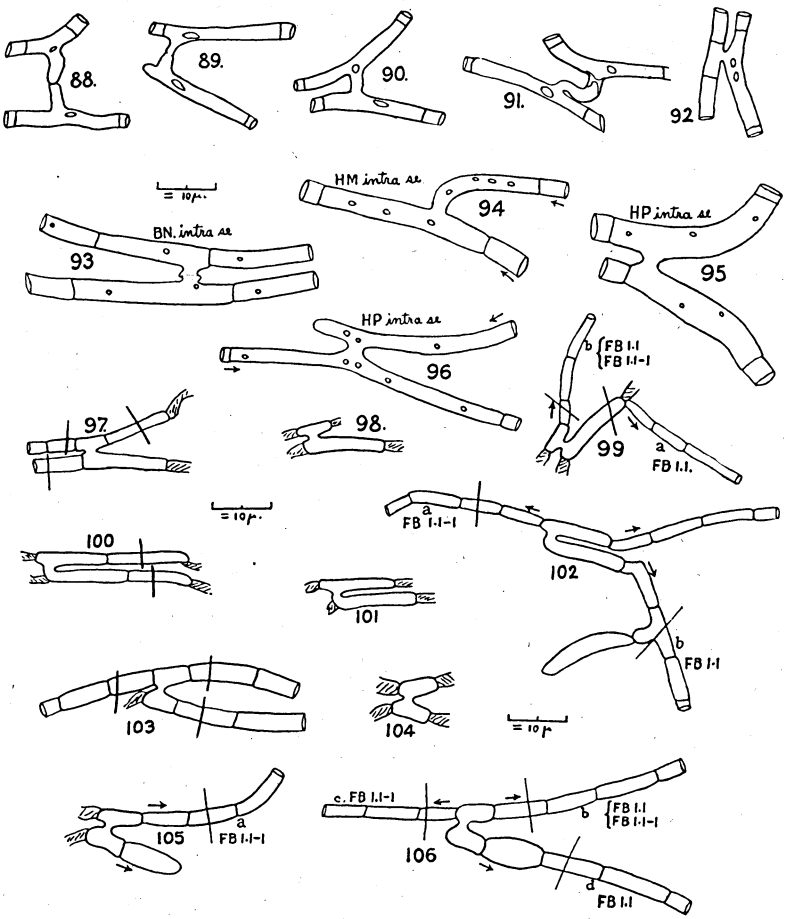


Fig. VI. Fusion cells

All drawings were made with the aid of a camera lucida using a Leitz microscope and a 1/12th oil immersion objective, a 12x eyepiece and a tube length of 170 mm.

88-92. Stages in the formation of fusion cells in $FB_{1.1}$. Notice in No. 92 the migration of one nucleus into the other half of the fusion cell.

93-96. Cases of fusion cells formed *intra se* in *H. pedicellatum, H. monoceros,* and *Helminthosporium sp.* (BN). Notice that the nuclear content is evenly divided between the two halves except in No. 96.

97-99. Fusion cell 4. No. 97 as first observed. No. 98 after isolation. No. 99 some twelve hours after isolation. The black lines indicate the cuts made on isolation.

100-102. Fusion cell 9. No. 100 as first observed. No. 101 after isolation. No. 102 some twelve hours after isolation. The black lines indicate the cuts made on isolation.

103-106. Fusion cell 13. No. 103 as first observed. No. 104 after isolation. No. 105 some twelve hours after isolation. No. 106 some eighteen hours after isolation. The black lines indicate the cuts made on isolation.

In Figure VI are shown the series of drawings made during the manipulation of three of the fusion cells, namely, Nos. 4, 9, and 13. In No. 4 (Fig. VI. 97-99) both parent strains were obtained, the one $FB_{1.1}$ by the isolation and subsequent growth from hypha "a," and both $FB_{1.1}$ and $FB_{1.1-1}$ by the growth from hypha "b," and subsequent single spore isolation. In No. 9 (Fig. VI. 100-102) strain $FB_{1.1-1}$ was produced by the growth of hypha "a" and strain $FB_{1.1}$ from a conidium formed on hypha "b."

Table 8

Production of Hyphal Fusions in Fusarium spp. at About 17° C.

Description of data	Parentage of 2-celled hyphal pairs								
	FA_2 × FA_2	FA_2 × $FB_{1.1}$	FA_2 × $FB_{1.1-1}$	$FB_{1.1}$ × $FB_{1.1}$	$FB_{1.1}$ × $FB_{1.1-1}$	$FB_{1.1}$ × FV	$FB_{1.1-1}$ × $FB_{1.1-1}$	$FB_{1.1-1}$ × FV	FV × FV
Total pairs of 2-celled hyphae put together ..	37	51	48	30	100	42	22	39	27
Total fusions that could have been produced	74	102	96	60	200	84	44	78	54
Total perfect hyphal fusions produced	51	4	0	47	153	16	31	0	40
Percentage of possible total of fusion cells...	69	4	0	78	76	19	70	0	74

In No. 13 (Fig. VI. 103-106) growth took place from three parts of the fusion cell, and accordingly isolations were made from these parts. At "a" after 12 hours growth a hypha was produced and, on isolation and subsequent growth, this gave strain $FB_{1.1-1}$. The fusion cell was then left for a further six hours and isolations were again made. From the hyphae "c" and "d" strains $FB_{1.1-1}$ and $FB_{1.1}$ were respectively isolated; from the hypha "b," both strains $FB_{1.1}$ and $FB_{1.1-1}$ were obtained after later single spore isolation.

Clearly the interpretation for such mechanical mixtures of both parents in the growth from fusion cells is uncertain in view of the paucity of evidence. These mixtures might be due to saltation, to contamination from the parent colonies, or to the first few cells produced retaining both potentialities. Saltation appears to be the least likely of the three causes. The reason for this suggestion is that only one case of saltation in Fusarium occurred during the whole year on Brown's medium; further, that no similar change took place in the equal number of control experiments; and that the suggested change was the same in all three cases. The reason that contamination is not thought a likely cause for these mechanical mixtures is that the agar blocks, isolated as

Table 9

Number of Successfully Isolated Fusion Cells of Fusarium spp. with Their Origin and Progeny

Parent strains	Total fusion cells successfully grown	Progeny isolated								
		New forms	Both parents	$FB_{1.1}$ and mixture	2nd parent and mixture	Mixture only	FA_2 only	$FB_{1.1}$ only	$FB_{1.1\text{-}1}$ only	FV only
$FA_2 \times FA_2$	7	—	—	—	—	—	7	—	—	—
$FA_2 \times FB_{1.1}$	1	—	1	—	—	—	—	—	—	—
$FB_{1.1} \times FB_{1.1}$	8	—	—	—	—	—	—	8	—	—
$FB_{1.1} \times FB_{1.1\text{-}1}$	14	—	6	1	2	—	—	2	3	—
$FB_{1.1} \times FV$	5	—	1	—	—	—	—	4	—	—
$FB_{1.1\text{-}1} \times FB_{1.1\text{-}1}$	4	—	—	—	—	—	—	—	4	—

controls, uniformly gave no growth, and that elaborate precautions were taken to guard against contamination (see Section II, Procedure, p. 30). It is considered, however, that no conclusion can be arrived at until further examples and satisfactory cytological preparations have been made.

Discussion

While a considerable amount of space has been taken up in describing the formation of hyphal fusions and the manner in which a method was evolved for obtaining fusion cells, the main interest lies in the progeny produced from the isolated fusion cells.

First, it must be admitted that thoro mixing or blending of the two cytoplasms in the isolated fusion cell is presumed to have occurred. Such an assumption is not altogether justified by the observations on nuclear migration in the fusion cell, or by the passage of granules from one component to the other, because it is entirely possible for two cytoplasms, or colloidal systems, to mingle in a cell without mixing. However, in our present state of knowledge it seems best to make this assumption.

It is considered that the method evolved made certain the parentage of the fusion cells. In addition, the high-power microscopical examination ensured true fusion and provided against more than the fusion cell, or a particular hypha, as the case may be, being isolated. Further, it is thought that the occurrence of mechanical mixtures of the two parents alters in no way the main observation, which is that no change in cultural characters took place despite the association, as shown by the cytological preparations, of the two nuclei from different parents in the one fusion cell.

This observation is considered sound because the 31 cultures from the 20 fusion cells treated all proved on testing to be exactly the same as one or the other of their parents. If there had been a cytoplasmic difference between the parents, then some of the 31 cultures should have been either a new or an intermediate form. Altho this result is based upon negative evidence, it is believed that the conclusion is sound, namely, that the difference between the strain $FB_{1.1}$ and its saltant $FB_{1.1-1}$ is not a cytoplasmic difference.

GENERAL DISCUSSION

In the preceding discussions in this paper, it has been concluded that, certainly in the case of Fusarium spp. and probably in the case of Helminthosporium spp., there is good evidence against the occurrence of heterocaryosis in these strains. Further, the evidence appears sound that in Fusarium spp. the difference between a saltant and its parent

strain is not of a cytoplasmic nature. Thus two of the possible methods by which variation can take place do not occur in these strains.

The classification of variations—using the term in a general sense—has produced considerable controversy, and the terms used for describing sorts of variations have been used and still are frequently used in a loose sense.

Baur (1) in 1914 classified variations under three headings: (1) modifications, non-heritable conditions caused by environmental conditions; (2) combinations, heritable differences caused by segregation and recombination of hereditary units of two kinds, (a) normal segregation and recombination, (b) aberrant segregation and recombination; (3) mutations, heritable differences that do not depend upon segregation or recombination.

Brierley (5), in 1926, classified the available cases on the basis of: (1) modification, non-heritable differences caused by the unequal influence of different conditions and varying immediately with the conditions; (2) continuous variations, heritable differences characterized by the gradualness of the change through successive generations, i.e., subculture generations; (3) discontinuous variations, heritable differences characterized by the suddenness of their appearance.

Both these authors, especially Brierley, used for the basis of their classifications the type of phenomena observed rather than the nature, or cause, of the change itself. It is improbable, however, that the basis could have been otherwise at the time the schemes were composed.

In Europe the term "mutation" has been used in the sense in which Baur used it, but in America it has been used to describe sudden changes without reference to whether the change was in Baur's sense a combination or a mutation. In 1922, Muller (38) realized this and suggested that it would be best to confine our use of mutation to one coherent class of events—that is, to real changes in the gene.

However, it is difficult to be certain whether a sudden change is due to gene mutation or chromosomal aberration, and it is thought by many that mutation should include not merely gene changes but also chromosomal aberrations. Bridges (3) has further subdivided chromosomal aberrations, but fungal genetics has not yet reached a stage when such subdivision is of value.

Such classifications as these require modification in the case of fungi, for Burgeff (13), as mentioned in the Review of Literature, has clearly shown that by mixing the cytoplasm and the nuclei of two strains of *Phycomyces nitens* he was able to synthesize new strains. He used the term "mixochimaera" for such a product. The genetic implications of his observations lie in the similar potential changes which could be brought about by hyphal fusions or anastamoses. These po-

tential changes might be in the direction of the separating out from pre-existing complexes, or the building up of new forms from pre-existing forms. In addition to commenting on the above possibilities, Brierley (5) also pointed out that cell and nuclear divisions are not correlated in fungi as they are in higher plants, and hence that hyphal fusions could more easily play such a rôle.

While the examples of the educability of fungi described by Marshall Ward (47), Salmon (42), and others have not, in general, been substantiated by later workers, e.g., Stakman (44, 45); Hammarlund (28) considered that he had been able to infect injured leaves of barley with *Erysiphe graminis tritici*. Uninjured plants, however, were not infected. Consequently, in view of the ease of contamination in the powdery mildews, the impossibility of laboratory culture, and the presence of a sexual stage in their life history, it appears that one should retain a mental reservation on the possibility of such a phenomenon. Another reason for such a viewpoint is that *a priori* one would suggest that a capacity for educability would depend on a cytoplasmic change, and there is evidence of this from the work of Jollos on Infusorias (32), Harder on Basidiomycetes (30), and Newton *et al.* on the rusts (39).

Jollos (32), in 1920, described cases of what he called "Dauermodifikationen" in Paramecium. These changes, which he attributed to cytoplasmic inheritance, were produced by growing the organisms in arsenic and calcium containing media. They were characterized by an increased tolerance (as measured by the rate of vegetative reproduction) to the medium. The changes persisted for some time after the source was removed, and did not generally persist through conjugation. Caldis and Coons (14) claim to have obtained similar results with achromatic variations in some fungi.

Harder (30) was able to separate the associated nuclei in the diploid stage of the Basidiomycete, *Pholiota sp.*, and found that for a time the uninucleate haploid strain thus produced showed attempts at reproducing the diploid morphology. Eventually this strain became the normal haploid strain. He attributed this result to the hanging over of a substance in the cytoplasm formed in the diploid stage. On the other hand, in the rusts, Newton *et al.* (39) obtained results similar to those of Gairdner (26) in flax. In both these cases apparently certain characters were transmitted by one parent only, the reciprocal cross not showing the character.

Against this positive evidence, in the oat smuts Dickinson (19, 20) could find no evidence of cytoplasmic influence. Christensen (17), using the corn smut, stated that his results were inconclusive. In addition, Brown (10) considered that his saltations in Fusarium were not Dauermodifikationen. Stakman *et al.* (44, 45) were never able to obtain

any evidence suggesting the possibility of educability in *Puccinia graminis*. Brierley (6), however, seems not quite convinced by his experiments with Botrytis that fungi can not be "educated."

It seems, therefore, that the work on cytoplasmic inheritance mentioned above necessitates considerable caution. One can not, as has often been done, ignore such evidence. Consequently it is thought that any classification of variation in fungi based upon the nature or seat of the change, has to include not merely combinations and mutations in the Baurerian sense, but also mixochimaeras and cytoplasmic inheritance. Such a classification may be composed as follows:

Eco-variants—non-heritable changes due to the environment.

Plasma-variants—heritable changes due to alterations in the cytoplasm.

(a) semi-permanent—"Dauermodifikationen."

(b) permanent—cytoplasmic inheritance.

Caryo-variants—heritable changes due to alterations in the nuclear content.

(a) Mixochimaeras—heritable changes due to the separation or combination of nuclei of different types.

(b) Mutations—(1) chromosomal aberrations—chromosome changes in the number, arrangement, etc., of the whole chromosomes or parts of chromosomes.

(2) gene changes—changes concerned only with a single gene.

If, with such a classification, the results of this investigation are considered, certain conclusions appear inevitable. As changes do occur in some of the strains used, and as such changes are permanent and are not concerned with the environment, then such changes are not Eco-variants. As no change was obtained after fusion and separation between the parent strain and the new type, the seat of the change must be in the nucleus, i.e., these changes are not Plasma-variants. As these changes have occurred after an uninucleate stage has been isolated, that is, in the progeny of a single nucleus, they can not be due to an assortment of different kinds of nuclei, that is, they are not Mixochimaeras. Therefore, these changes must be due to some alteration in the nucleus, that is, a mutation, but whether such an alteration be a chromosomal aberration of some sort or a gene change can not as yet be determined.

Apart from these conclusions on the nature of saltation and the rôle that hyphal fusions have been suspected of playing in the origin of new forms, for which latter no evidence has been found, there is the somewhat academic question of the origin or nature of hyphal fusions in themselves. Naturally a relation between the cell fusion in the sexual process, and the cell fusion on which these experiments have been made, may be suspected; altho no relation can be presumed until it is

proved. There is the evidence that suggests that the attraction (and resulting fusion?) of sexually different hyphae is due to a chemotropic stimulus between the two hyphae. Sometimes one, sometimes both, are active. In these hyphal fusions, there appears strong grounds for considering that a diffusion of some substance from one or from both hyphae is responsible for the attraction (and resulting fusion?). But for the fusion following the attraction the evidence in both sexual and hyphal fusions is negligible or non-existent. It is not known, for example, whether, if two active hyphal tips were made to grow straight toward one another, fusion would take place. Yet that appears to be exactly how a fusion cell is formed. Further, there is the clear-cut quality of fusion in the sexual process, for example, no fusions *intra se* in the smuts, numerous fusions with the opposite type (19). In hyphal fusions, so far as is known, fusions take place most frequently between closely related cells, tho there is some evidence (Matsumoto, 36, etc.) of their production in less closely related cells.

Buller's (12) point of view of hyphal fusions leads him to a straight teliological explanation, which is probably correct. . . . Such an explanation is not entirely satisfying. However, until further experimental work has been done, it must suffice.

SUMMARY

The organisms used in these investigations were *Helminthosporium pedicellatum* Henry; *H. monoceros* Drec; *H. sp.* (Brachysporium type); *Fusarium fructigenum* Fries; *F. vasinfectum* Atk.

It was found that all the numerous nuclei in the conidia of these Helminthosporium species originate from a single nucleus, and that each cell in both the mycelium and conidia of these Fusarium species contain one nucleus.

When cells or conidia, either containing one nucleus or arising from one nucleus, were isolated, they invariably gave rise to colonies similar to those of the parent strains.

After such isolation, it was found that these strains would, under appropriate conditions, give rise to saltant strains.

It is therefore concluded that heterocaryosis is not responsible for such saltations in these species.

Fusion cells were formed between pairs of cells of two contrasting saltant strains of *Fusarium fructigenum*. It is presumed that during the fusion cell condition the two cytoplasms were thoroly mixed. On isolation from the subsequent growth of such fusion cells, the cultural characters of the two parent strains were found unchanged.

It is therefore concluded that the difference between such strains is not cytoplasmic.

Since heterocaryosis and cytoplasmic inheritance have been disproved, it is concluded that the nature of saltation in these strains of *Fusarium fructigenum* is a change in the nucleus, that is, a mutation.

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