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Hyphal fusions in the wood-rotting fungus *Schizophyllum commune*

I. The effects of incompatibility factors*

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SUMMARY

Effects of the incompatibility factors in *Schizophyllum commune* Fries on the process of hyphal fusion are described. A role for the A incompatibility factor in hyphal fusion is indicated. Matings between strains with different mating types have higher fusion frequencies than matings between strains with the same mating types. Evidence is presented that the differences in fusion frequencies are not due to genetical factors other than mating types. When two strains of different mating types are grown in the same culture plate, but separated by a cellophane membrane, the strains are altered in some unexplained manner in such a way that even matings between strains of the same mating type have a higher fusion frequency than occurs in matings between compatible strains not so treated. Matings leading to the formation of common-B and dikaryotic mycelia have comparable fusion frequencies while those leading to the formation of common-A mycelia have a far lower frequency of fusions. It has been demonstrated that high fusion frequency is associated with heterozygosity at the A locus. It is suggested that a repression–derepression mechanism involving a cell wall degrading enzyme or enzymes may be involved in the regulation of hyphal fusion.

1. INTRODUCTION

In *Schizophyllum commune* Fries, a heterothallic basidiomycete displaying the tetrapolar pattern of sexuality, the type of reaction when two homokaryotic mycelia are brought together is determined by the A and B incompatibility factors (Papazian, 1950; Raper & Miles, 1958). A dikaryotic mycelium is obtained only when the incompatibility factors are different at both loci. Different heterokaryotic reactions are observed if the incompatibility factors are the same at one locus but different at the other, and both common-A and common-B heterokaryons have been unequivocally shown to occur (Papazian, 1950; Raper & SanAntonio, 1954; Parag & Raper, 1960). The prerequisite for any kind of reaction, however, is

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the occurrence of hyphal fusion between the confronting hyphae. A great deal has been done to unravel the mystery of the incompatibility factors (Raper, Baxter & Middleton, 1958; Raper, Baxter & Ellingboe, 1960; and Parag, 1962) and models have been proposed to pinpoint the sites of action and the mode of operation for each factor (Raper, *Genetics of Sexuality in Higher Fungi*, 1966, p. 213). Raper has presented a pattern wherein A and B factors control and regulate events, taking place after hyphal fusion, leading up to the establishment of a dikaryon and clamp-connexion formation. A supposition, however, seems to be that mating type comes into play only after hyphal fusions have taken place to pave the way for nuclear migration. It is possible, however, that mating type differences may be important for the immediately preceding events. Interest in this latter possibility was aroused when, during the course of studies of environmental effects upon hyphal fusions, it was observed that fusion frequencies were far lower when the same strains were confronted with each other than when matings were between two compatible strains. Subsequently it was observed that when different strains of the same mating type were mated, the frequency of hyphal fusion was low. The present paper demonstrates a relationship between mating type and frequency of hyphal fusions in *S. commune*.

2. MATERIALS AND METHODS

The strains used are shown in Table 1. Cultures used as sources of inocula were grown on agar plates. The composition of the medium was: 20 g dextrose, 2 g peptone, 0·46 g KH_2PO_4, 1 g K_2HPO_4, 0·5 g $MgSO_4.7H_20$, 20 g Bacto agar and 1 l. of distilled water. The medium was autoclaved for 20 min at 121 °C under 15 lb pressure.

Table 1. *Strains used*

Strain no.	Incompatibility factors	Morphology
699	41/41	+
2233	41/41	+
2250	41/41	+
2275	41/41	+
3054	405/405	+
3064	406/406	+
3074	42/42	+
3075	42/41	+
3076	41/42	+

+ wild-type morphology.

Cultures incubated at 30 °C for 6 days served as inocula for making confrontations between mycelia. The inocula were taken from the margins of the colonies so as to have mycelia of approximately the same age. The inocula were punched out from the solid agar cultures by means of a punch with a rectangular opening, 5 mm long and 2 mm wide. Use of rectangular inocula allowed more area for hyphal contacts and growth was unidirectional for at least 2 days, the time period for which the preparations were incubated.

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The method of making the preparations was a slight modification of that of Robak (1942). Van Tieghem cells were prepared by fusing 22-mm square glass plates, with holes of 15 mm diameter and 2 mm depth (obtained from Arthur H. Thomas Co., Philadelphia), into glass slides (75 × 25 × 1 mm).

The inocula were lifted from the plates on the tip of a sterilized scalpel and placed upon a flamed 22 mm square no. 1 coverslip, mycelial side down. The two inocula between which the confrontation was made were kept about 5 mm apart. The coverslip with the inocula was then placed on top of the Van Tiegham cell. Before sealing the cell, however, a very small amount of water was introduced into the cell chamber to prevent desiccation. The preparations were incubated at 30 °C unless stated otherwise. Incubation was for a period of 48 h, the time period which gave the most satisfactory growth for good observations. Incubations for lesser periods gave too sparse growth, while longer periods gave too profuse growth for the hyphae to be traced back to their origins.

Observations were made with a compound microscope at magnifications up to 1400×. The observational area under the microscope was limited to the space between the two inocula where two hyphae, one from each inoculum, met with each other. Such a meeting was called a 'contact' and meant any point of contact between the two hyphae, either sideways, tip to tip, or under or above each other. When any contact was suspected, it was examined carefully first with the low and then with the high power objective. The two hyphae were traced back to their respective origins and only those contacts were taken into account in which the two contacting hyphae could be clearly traced back to their origins. This established that the contact originated from the two confronting inocula. In each experiment the number of all such contacts was counted in a total of 60 cells and recorded. This number of cell preparations was determined by the time factor and physical limitations. Each experiment was set up in duplicate.

Observations were possible with only those hyphae which grew in contact with the glass surface, while the aerial hyphae escaped examination. This slight disadvantage, however, can be discounted because not many aerial hyphae are given out by inocula prepared in the manner described.

Hyphal fusions observed with the high dry objective were scrutinized carefully under the oil immersion objective to establish firmly the validity of a fusion.

In order to determine experimentally if a diffusable substance is produced by one strain which might induce hyphal fusions to occur in greater frequency in another strain, one strain was inoculated on the agar and then covered by an autoclaved cellophane membrane. The inoculum of the inducing strain was then placed on top of the membrane, slightly to one side of the inoculum beneath the membrane. Plates were incubated at 30 °C for 6 days at the end of which period the cellophane membrane with the growing culture of the inducing strain was discarded. The colony growing on the agar (hereafter referred to as the induced strain) served as the source of inocula for preparations made as described earlier.

The effect of mating type and treatments upon hyphal fusion frequency was

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analyzed statistically by use of contingency tables and the χ^2 test as a test for independence. In essence, this is given in Fisher (1948) as a hierarchical χ^2 test.

3. RESULTS

Hyphal fusions were observed in most matings irrespective of incompatibility factor differences. The relative frequencies, however, of hyphal fusions in matings between strains of the same incompatibility factors and those between strains having different factors varied markedly from each other. The same types of fusion were observed as those listed by Buller (1933). Tip-to-tip and peg-to-peg fusions were relatively easily distinguishable, but it was not always possible to see a peg in the so-called tip-to-peg type of fusion; therefore, all such fusions were classified as tip-to-side type. Fusions of the tips to the sides of the hyphae appeared to be the most prevalent type of fusion. Within a short distance of about 10 μ, the two approaching hyphal tips show an attracted growth towards each other (Plate 1, figs. 1–3). At greater distances, the two keep growing in their respective directions. Attracted growth was only observed between hyphal tips in matings between compatible mating types.

(i) *Fusion frequencies of compatible and non-compatible matings*

Matings were made both between the same strains, between compatible strains, and between different strains with the same mating types at different temperatures. Table 2 shows fusion frequencies in matings between the same strains and between compatible strains at different temperatures. It can be seen clearly that there is little or no effect of temperature, and consequently the data can be added between temperatures to test for differences between crosses. Of interest in the present study is the fact that many more hyphal contacts (from three to ten times as many) were made before a fusion occurred in matings between the same strains (e.g. 699×699) than when matings were made between different mating types (e.g. 699×3054). The χ^2 analysis (Table 2, bottom) shows that there is a significant difference in hyphal fusion frequency between groups (compatible strain confrontations compared with same strain confrontations) but there is no significant difference within groups; i.e. hyphal fusion frequency is significantly higher in confrontations between compatible strains than in confrontations between hyphae of the same strain.

That the differences in fusion frequencies were not caused by factors other than mating-type differences was made evident by the results obtained in matings between different strains having the same mating types (Table 3). The fusion frequency is low in all cases and about the same as for similar crosses in Table 2.

(ii) *Fusion frequencies of induced and non-induced strains*

When strains were induced by compatible mating types as described in Materials and Methods and these induced strains were confronted in compatible pairings, the fusion frequencies were significantly higher than those of compatible matings of

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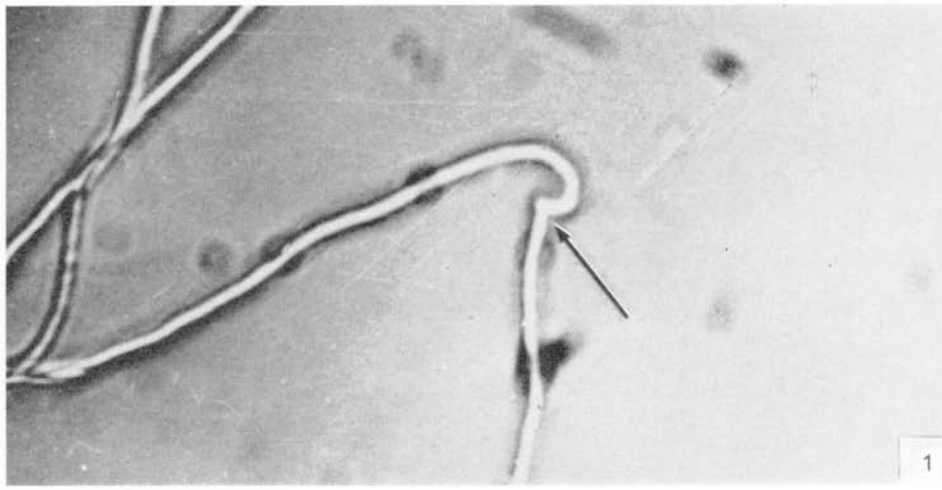

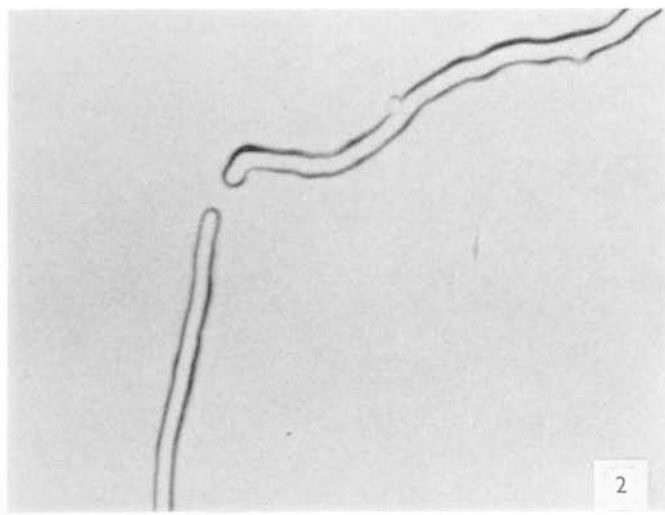

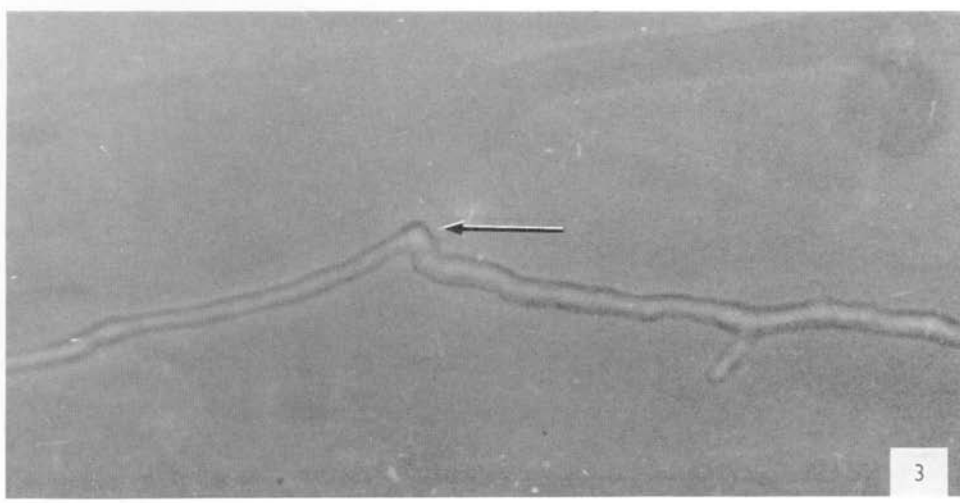

Figs. 1 and 3 show tip-to-tip type of hyphal fusion. In Fig. 2 a hyphal tip is seen bending towards another. Note in Fig. 1 the directed growth of one hyphal tip towards and fusion with another hyphal tip. The phenomenon is also observed slightly in Fig. 3.

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Table 2. *Hyphal fusions in matings between the same strains and between compatible strains at different temperatures*

Cross	Temp. (°C)	No. of contacts	No. of fusions	Fusions per contact × 1000
699 × 3054	20	195	7	36
	25	232	7	30
	30	234	9	38
699 × 3064	20	382	14	37
	25	303	9	30
	30	370	12	32
699 × 699	20	219	1	4
	25	215	1	4
	30	345	5	14
3054 × 3054	20	347	0	< 3
	25	489	2	4
	30	284	1	4
3064 × 3064	20	208	0	< 5
	25	328	0	< 4
	30	358	2	6

Analysis:

	Fusions	Non-fusions	Total fusions	Total non-fusions	Total
Compatible group	23	638	58	1658	1716
	35	1020			
Same strain group	7	772	12	2781	2793
	3	1117			
	2	892			
Total	70	4439	70	4339	4509

	χ^2	D.F.	P
Total	62·17	4	< 0·001
Between groups	60·54	1	< 0·001
Within groups	1·63	3	0·65

Table 3. *Hyphal fusions between same mating types at* 30 °C

Cross	No. of contacts	No. of fusions	Fusions per contact × 1000
699 × 2250	292	3	10
699 × 2233	288	2	7
699 × 2275	200	0	< 5
2233 × 2250	264	1	4
2233 × 2275	198	0	< 5
2275 × 2250	246	1	4

self-induced hyphae—that is hyphae in which the inducing treatment involved the use of a single strain placed on both sides of the cellophane membrane (Table 4). Significant differences are revealed when non-induced strains are compared with induced strains (in both same strains and compatible confrontations, Table 4 at bottom). No significant differences were obtained between the fusion frequencies when compatible, induced and same strain, induced confrontations were compared.

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These observations lead us to believe that some substance is released into the medium which in the presence of incompatibility differences, produces much higher fusion frequencies.

Table 4. *Hyphal fusions between induced hyphae at* 30 °C

Cross*	Description of cross	No. of contacts	No. of fusions	Fusions per contact × 1000
699/*3054* × 3054/*699*	Compatible, induced	64	6	94
699/*3064* × 3064/*699*	Compatible, induced	114	8	70
699/*699* × 3064/*3064*	Compatible, self-induced	263	9	34
699/*699* × 3054/*3054*	Compatible, self-induced	190	5	26
699/*3054* × 699/*3054*	SS†, Induced	64	4	62
699/*3064* × 699/*3064*	SS, Induced	85	6	70
3054/*699* × 3054/*699*	SS, Induced	78	5	64

* Crosses were made between strains shown here to the right of the slanting line, and in italic figures. Strains to the left of the slanting line were used as inducers.

† Same strains.

Analysis:

	Fusions	Non-fusions	Total fusions	Total non-fusions	Total
Compatible, induced group	6 8	58 106	14	164	178
Compatible, self-induced group	9 5	254 185	14	439	453
SS, induced group	4 6 5	60 79 73	15	212	227
Total	43	815	43	815	858

	χ^2	D.F.	P
Total	8·45	6	0·20
Between groups	7·77	2	0·02
CI vs. SS, I	0·24	1	0·62
C, Self vs. SS, I + CI	7·44	1	< 0·01
Within groups	0·68	4	> 0·95

(iii) *Incompatibility factors and hyphal fusions*

Noting that differences in the incompatibility factors noticeably enhanced the hyphal fusion frequencies, we next tried to determine if one of the two mating-type factors exerted itself more strongly in the phenomenon of hyphal fusion. Matings were made between four strains in such combinations as to give two common-A, two common-B and two compatible matings (Table 5). The fusion frequencies of the compatible and common-B matings were much higher than the fusion frequencies of common-A matings. When the results were arranged to compare those confrontations involving common-A factors in one group and uncommon-A factors in another, significant differences were obtained (Table 5, bottom). These data suggest that a difference in A factors leads to a greater number of hyphal fusions.

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Table 5. *Effects of incompatibility factors on hyphal fusion*

Cross	No. of contacts	No. of fusions	Fusions per contact × 1000
Compatible			
699 × 3074	149	5	34
3075 × 3076	369	10	27
Common-B			
699 × 3075	264	9	34
3074 × 3076	159	4	25
Common-A			
699 × 3076	363	3	8
3074 × 3075	208	1	5

Analysis:

Group	Fusions	Non-fusions	Total fusions	Total non-fusions	Total
Compatible	5	144	15	503	518
	10	359			
Common-B	9	255	13	410	423
	4	155			
Common-A	3	360	4	567	571
	1	207			
Total	32	1480	32	1480	1512

	χ^2	D.F.	P
Total	9·59	5	0·09
Between groups	8·91	2	< 0·02
Compatible vs. Common-B	0·02	1	0·88
Common-A vs. Common-B + Compatible	8·88	1	< 0·01
Within Groups	0·67	3	0·88

DISCUSSION

While this study was focused on the events following contact of the hyphae, observations indicating a chemotropic activity of the hyphae have been made and it is of interest to note that such activities have been shown to occur in some other fungi (Buller, 1933). Hormonal mechanisms have more recently been studied by Bistis (1956, 1957) in *Ascobolus stercorarius* and by Raper (1951, 1957) in *Achlya* spp. The observation that fusions occur with a high frequency—even between strains with the same mating types—if each confronting mycelium has been induced by growing it in the presence of a strain of different mating type, indicates that some substance is released into the medium, leading to the higher fusion frequency. Such inducing effect, however, is difficult to explain.

The higher fusion frequency between strains of different mating types and the demonstration that genetic factors other than mating type did not cause any difference in fusion frequency indicates that mating type exerts an influence upon the process of hyphal anastomosis. The requirement for heterozygosity at the A locus for high fusion frequency is strongly indicated in this study by comparing

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fusion frequencies of common-A, common-B, dikaryon, and common-AB matings. This previously unsuspected role for the A factor in *S. commune* may be a fundamental one in the biology of the Basidiomycetes.

That genetic constitution may affect the fusion frequency was suggested by Pontecorvo (1946). Garnjobst (1953, 1955) produced evidence that genetic factors are involved in anastomosis as well as in the ability to continue growth as a heterokaryon in *Neurospora crassa*. It has been suggested that such a system of genetic control reduces the chances of frequent union between related strains. Reports of failure to establish heterokaryons in many other forms may be due to the presence of similar incompatibility systems. Leonian (1930), for example, failed to make what he called the 'Mixochimaera' between all but four of several mixtures of spores from two morphologically distinct colonies which arose from a single isolate of *Fusarium moniliforme*.

The process of dikaryosis (nuclear migration, conjugate division, and formation of clamp connexions) in tetrapolar species is under the control of A, or B, or both factors (Fulton, 1950; Takemaru, 1961). Sexual morphogenesis in *Schizophyllum commune* is a summation of two separate sequences of events regulated by the A and B incompatibility factors (Raper & Raper, 1966). In the sequential progression of dikaryotization, the nuclear pairing, conjugate division, and the formation of hook cells are under the control of the A factor. There is a similarity between the fusion of the hook cell with a bump on the penultimate cell and our tip-to-peg type of fusion. The known role of the A factor in the formation of the hook cell agrees with the present finding that the A factor influences the process of hyphal fusion. The nuclear distribution in common-A heterokaryons lends itself to support the hypothesis that different A factors are required for a higher fusion frequency. Out of 314 one and two-celled hyphal tips of common-A heterokaryons, only one had a migrant nucleus—i.e. a nucleus of the type possessed by the invading mycelium prior to the establishment of the heterokaryon (Raper & San Antonio, 1954). In the multicelled tips 90 % had uninucleate cells and only 6 % of these were migrant type nuclei. This low incidence of nuclear transfer may be attributable to low fusion frequencies. In summarizing the essential pattern of nuclear distribution in newly established common-A heterokaryons, Raper recognized a restraint exerted by the invaded mycelium upon the migrant nuclei. This restraint may well be the occurrence of fewer hyphal fusions and, therefore, a lesser number of channels for the nuclei to pass through.

The fusion event certainly involves the activity of cell wall degrading enzymes. Wessels & Niederpruem (1967) have shown increased R-glucanase activity (a cell wall degrading enzyme of *S. commune*) in common-A heterokaryons; however, an increase in the level of R-glucanase, although to a lesser extent, is also observed in common-B heterokaryons. The dikaryons also show a transient small increase in the level of the enzyme. The cell wall of *S. commune* is composed, in addition to R-glucan of S-glucan and a glucosamine containing polymer (Wessels, 1965); and, therefore, enzymic equipment for digesting the latter two components is necessary for a complete degradation of the cell wall at the point of contact of two

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fusing hyphal tips. Activities other than those resulting from the heterozygosity at the B locus may, therefore, reasonably be expected to play a role in the process of hyphal fusion. Since R-glucanase activity is highest in common-A matings, and since highest hyphal fusion frequencies are obtained in matings heterozygous for the A factor, it would be expected that cell wall degrading enzymes other than R-glucanase play an important role in the process of hyphal fusion.

That the incompatibility factors are involved in the process of hyphal fusion is clear from the present studies. In view of the possibility that an enzyme digesting the glucosamine containing polymer may play a role in the process, we might next consider the possibility that the A incompatibility factor is involved in the production of the enzyme. This may act in a manner analogous to the proposed repression–derepression model of Raper (1966) for the incompatibility factors. In this case a heterozygous condition at the A locus would derepress the enzyme; whereas, a homozygous condition would produce effective repressors of the enzyme.

The present studies have established certain facts concerning the genetic control of hyphal fusion and have permitted speculation about the details of the complete process. One aspect, the bringing together of hyphal tips, has not been studied experimentally. The biochemical events leading to the dissolution of the cell walls at the region of contact and the re-establishment of the cell walls and cytoplasmic continuity between the fusing hyphae have not been investigated directly. The fact that hyphal fusion, a very basic feature in the sexual process in filamentous fungi, is regulated in a predictable manner by genetic factors and is not merely decided by chance and environmental conditions has now been clearly established.

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