Genet. Res., Camb. (1968), **12**, *pp.* 305–315
With 1 *plate and* 3 *text-figures*
Printed in Great Britain

Growth rate and haploidization of *Aspergillus niger* on medium containing *p*-fluorophenylalanine*

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(*Received* 13 *May* 1968)

1. INTRODUCTION

A crude comparison of growth rates of haploid and diploid colonies of *A. niger* on complete medium (CM) and complete medium supplemented with *p*-fluorophenylalanine (FPA) led to the hypothesis (Lhoas, 1961, 1967) that FPA induces chromosome losses in the haploid and in the diploid, the successive losses in the latter leading to the formation of haploid nuclei. The work described here was undertaken to confirm the mechanism of this action. Three different approaches were followed: first the measurement of the dry weight of haploid and diploid colonies grown on CM and FPA, secondly the study of the growth curves of haploid and diploid strains on both media and, finally, the cytological observation of the region of active growth.

2. METHODS

General techniques were those described by Pontecorvo, Roper, Hemmons, MacDonald & Bufton (1953) and Lhoas (1961, 1967). All experiments were performed at 28 °C and CM was always supplemented with the growth factors required by the haploid strains.

(i) *Strains*

Two haploid strains were used: *anic3pab5* (fawn-spored haploid) and *omet6*; *his* (olive-spored haploid); the diploid studied was synthesized from them following Roper's (1952) technique. Mutant alleles are described in Lhoas (1967).

(ii) *Dry weight*

A few spores were inoculated in the centre of a piece of permeable cellophane membrane spread on the solid medium of a Petri dish. Seventy-two hours after inoculation, the thin layer of mycelium was carefully stripped off the cellophane and dried at 110 °C. for 48 h before weighing.

(iii) *Establishment of growth curves*

Clutterbuck & Roper's (1966) technique for single-layer culture was used; in order to standardize growth conditions, glass rings containing 5 ml. of medium were used and, to reduce evaporation the inoculated slide was left in a disposable

* The work was done in part at the Department of Genetics, The University, Glasgow.

https://doi.org/10.1017/S0016672300011897 Published online by Cambridge University Press

Petri dish through which photographs were taken. From the time of germination (about 8 h after inoculation) up to 40 or 50 h later, a photograph of the tip of the hypha germinating from a well-isolated conidium was taken every half hour. The length increase of the hypha during the time interval was measured on photographs at a final enlargement of $\times 700$.

(iv) *Determination and cytological observation of the region of active growth*

Except for the following points the method of Clutterbuck & Roper (1966) was used.

The region of active growth was determined from the growth of severed and control hyphae measured on camera-lucida drawings. The nuclei of single-layer cultures were stained in acridine-orange 0·1 mg./ml. in distilled water for about 2 min. For the observation of the nuclei, a HBO Li 200 mercury lamp, with Reichert E3 and SP3 filters, and a dark ground condenser were used. Camera lucida drawings of tip cells taken at random in the single-layer culture were made at an enlargement of $\times 1000$. The use of black paper and white pencil made the drawings much easier. The projected area of the nuclei was measured to the nearest μ^2.

3. RESULTS

(i) *Dry weight*

Table 1 gives the dry weight of haploid and diploid thin-layer colonies on CM and FPA. For each strain the ratio of dry weights on CM and on FPA agrees with the ratio of the diameter of haploid and diploid colonies grown on the same media (Lhoas, 1967); despite this similarity which was probably due to the growth conditions of the colonies measured for dry weight, namely thin layer growth on cellophane membrane, one concludes from the dry weights obtained that the difference between the two media, for normal or thin layer growth, cannot be assigned exclusively to a different type of branching on FPA, but is due at least partially to different growth rates on the two media.

Table 1. *Dry weight (mg.) of haploids and diploid 64 h after germination*

(Mean of five tests with S.E.)

Strain	CM	FPA	Ratio CM/FPA
Fawn haploid	$36{\cdot}64 \pm 0{\cdot}43$	$8{\cdot}18 \pm 0{\cdot}25$	4·5
Olive haploid	$42{\cdot}38 \pm 0{\cdot}85$	$10{\cdot}78 \pm 0{\cdot}38$	3·9
Diploid	$40{\cdot}32 \pm 0{\cdot}76$	$5{\cdot}60 \pm 0{\cdot}23$	7·2

(ii) *Growth curves*

The growth curves of the main hypha arising from a well-isolated conidium (Text-fig. 1) were drawn by the method of least squares from the length increase (the unit being 7 μ) per unit of time. For the curve of the diploid on FPA, the figures of the small plateau were included in the calculation of the ascending as well as of the descending slope.

For the olive haploid and diploid on CM, the curve for the stationary phase had

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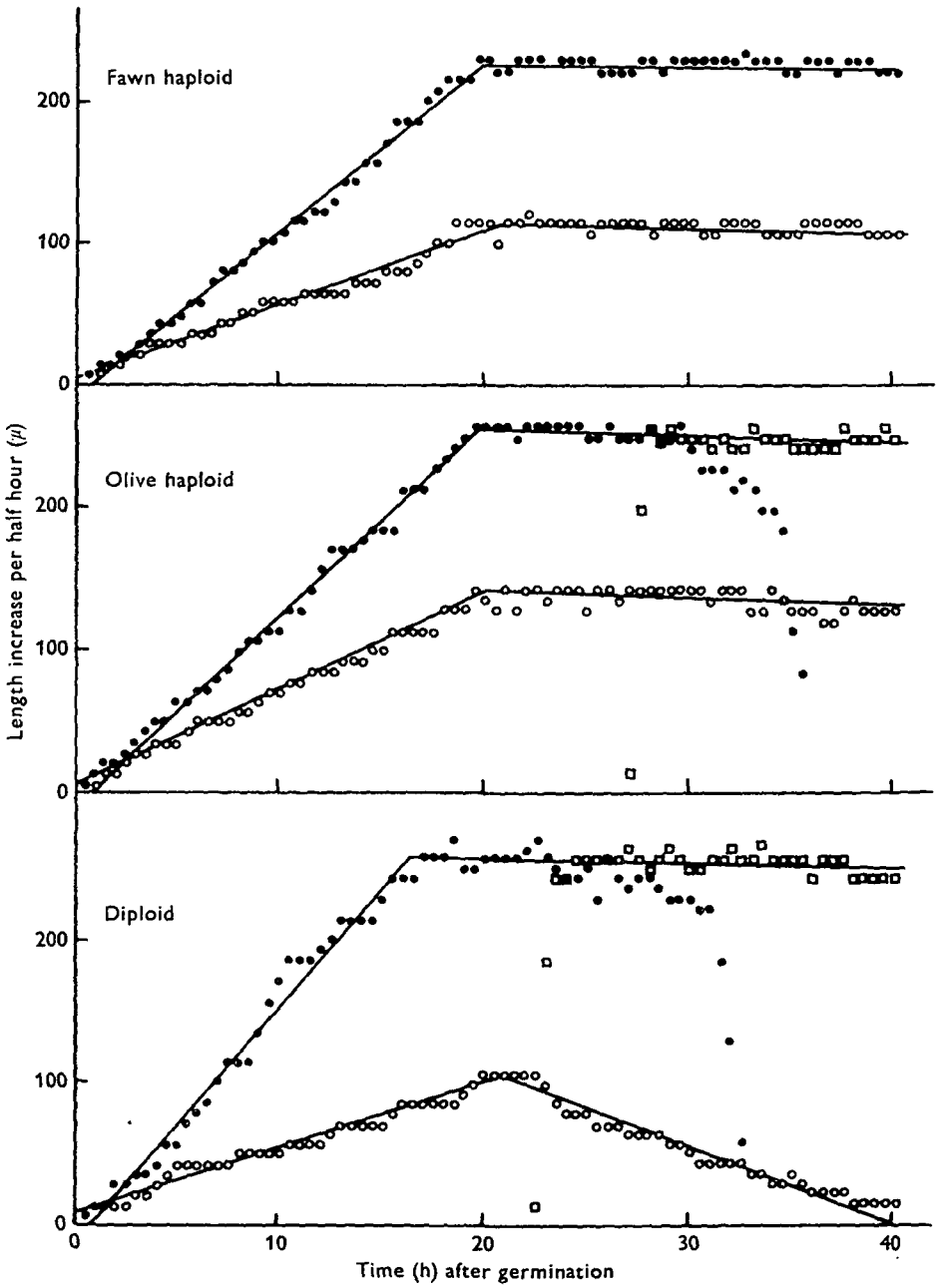
to take into account the fact that the main hypha was overtaken by a primary branch less than 40 h after germination. So that the curve gives a true picture of the mean growth of the colony, the growth rate of the branch was substituted for the growth rate of the main hypha as soon as the former was greater; this corre-



Text-fig. 1. Growth curves of haploid and diploid hyphae on CM (●—●: main hypha; □——□: primary branch) and on FPA (○——○).

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sponded more or less to the time at which the tip of the branch overtook the tip of the main hypha. The picture of the growth rate thus presented is that of the colony as a whole since the hypha being measured is always at the margin of the colony.

On CM, for the diploid as well as for the haploids, the length increase of the hyphae per time unit goes up linearly from the time of germination up to more or less 20 h later; once the maximum has been reached, the elongation per time unit remains almost constant till the end of the experiment. These linear growth curves agree with the findings of Smith (1924) in *Botrytis cinerea*.

On FPA, there is a striking difference between the haploids and the diploid. For the haploids, the values on FPA are about half the corresponding values on CM for the slope and for the plateau; in other words, throughout the length of the experiment, the growth rate on FPA equals, at any given time after germination, about half the value of the growth rate on CM at the same time. For the diploid, however, while the same is true for the ascending slope, although the ratio is smaller (one-third instead of one half), the part corresponding to the stationary phase of the haploids consists of a linear descending slope, much steeper than that which is found in the haploids: after having reached a maximum, the growth rate goes down quickly and regularly to only about 15 μ per half hour; this minimum growth rate was found constant up to 50 h after germination, i.e. for more than 10 h.

These growth curves explain the difference between the measures of the dry weight and of the diameter of the colonies on both media.

Each of the experiments reported here has been repeated once, but the curve of the diploid on FPA has been made three times. All results agreed with those given in Text-fig. 1, except that in one of the experiments on FPA the diploid produced a hypha growing at the steady rate of a haploid on FPA; similar hyphae could be shown (see the following section) to contain haploid-sized nuclei.

(iii) *Cytology of the region of active growth*

(*a*) *The region of active growth*

In Text-fig. 2, the elongation shown by severed hyphal tips of different lengths is compared with the increase in length of uncut hyphae. This was done with the fawn haploid. It is clear that growth is reduced when the severed hyphal tip is less than 1·2 mm long; as the length of the tip cell varies between 0·7 and 1·3 mm, this cell seems to be responsible for the growth. In this respect, *A. niger* behaves like *A. nidulans* except that in the latter the region of active growth is shorter than half what it is in the former, just as is the length of the hyphal tip cell. It is therefore the hyphal tip cell which will be studied for its content.

(*b*) *The distribution of nuclear size in hyphal tip cells*

Text-figure 3 gives the proportions of the nuclei of each size in the haploid, in the diploid after 20 h on CM (CM 20) and in the diploid after 20 and 40 h on FPA (FPA 20, FPA 40). The measuring method did not give significantly different results for the haploid on CM and on FPA. The length of the incubation period was determined by the growth curves: on CM and on FPA, the maximum growth

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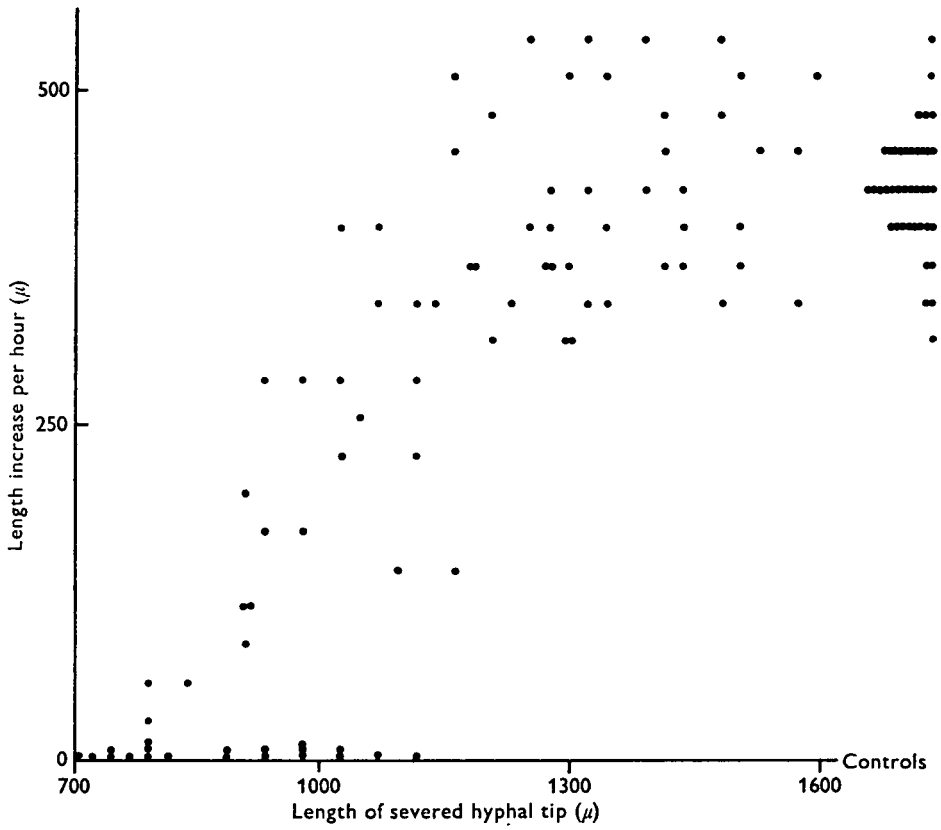
rate is reached 20 h after germination, the minimum for the diploid on FPA just before 40 h. For the haploid, 25 cells from 5 slides (5 from each slide) and, for the diploid, 49 cells (7 from 7 slides) were examined.

We notice first the normal curve of distribution of nuclear size for the diploid on CM; the diploid on FPA 20 has a very similar curve, although somewhat flattened; but a striking difference appears for FPA 40: the proportion of the 1 μ^2 nuclei increases and reaches the proportion of the 2 μ^2 nuclei. As the mode of the



Text-fig. 2. Growth of severed and control hyphal tip cells.

distribution of nuclei in haploid hyphae is 1 μ^2, this should be taken as the expression of a significant increase of haploid—or at least almost haploid—nuclei in the diploid after only 40 h on FPA.

There is no heterogeneity between slides except in the case of diploid FPA 40 where there is a slight heterogeneity [$F = 2{\cdot}41$; $F_{0{\cdot}95}(6,42) = 2{\cdot}32$]; the standard deviation of the mean area was calculated from pooled variance. Means of the nuclear projected area for diploid CM 20 ($2{\cdot}13 \pm 0{\cdot}04$) and diploid FPA 20 ($2{\cdot}13 \pm 0{\cdot}02$) do not differ, but are significantly different from the mean of diploid FPA 40 ($1{\cdot}60 \pm 0{\cdot}02$) as well as from the mean of haploid CM 20 ($1{\cdot}27 \pm 0{\cdot}01$). It is therefore evident that the 20 h treatment of the diploid on FPA is too short to bring a

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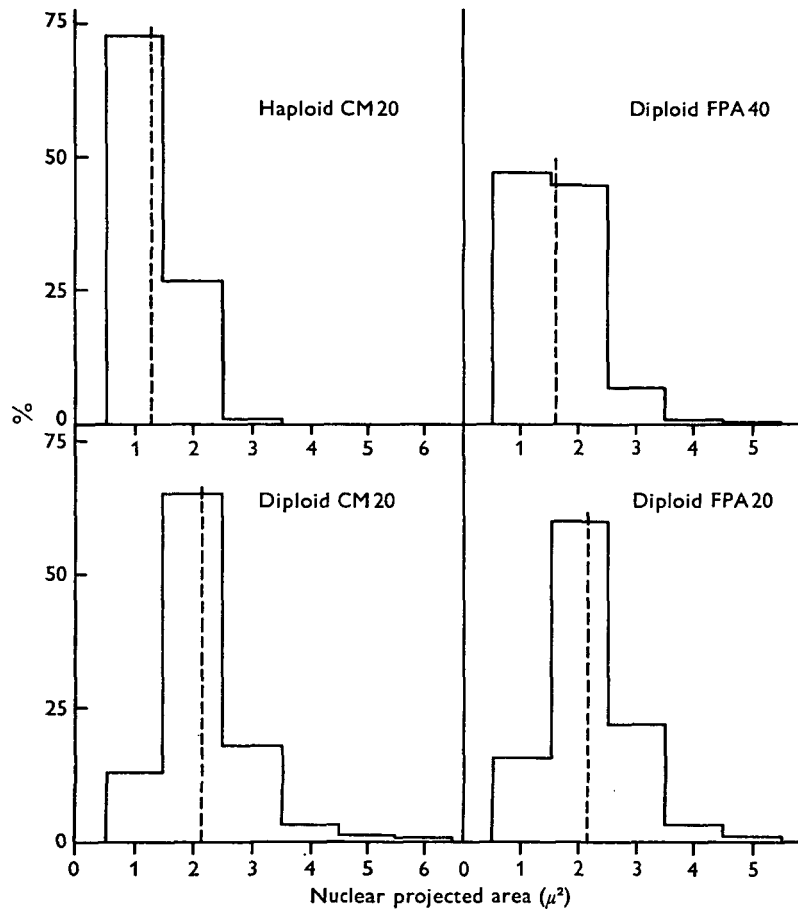
significant change in the distribution of the size of its nuclei, but that the 40 h treatment does, lowering the mean of the nuclear size to about half way between the means of the haploid and of the diploid on CM.

(c) *The proportion of micronuclei*

Table 2 summarizes the results of the cytological study of haploid and diploid on both media with respect to the number of normal nuclei and micronuclei. In addition, to the slides studied for the distribution of nuclear size, haploids FPA 20 and FPA 40 (5 cells from each of 5 different slides) were examined. As there was no difference on CM between 20 and 40 h, only the values for the first are given.



Text-fig. 3. Nuclear area distributions of haploid and diploid on CM and on FPA. Broken lines indicate the mean nuclear area.

Micronuclei are those fragments of nuclear material which could not be drawn with the camera lucida and had to be merely located on the drawings by a spot, while all the normal nuclei, already reported on in the previous paragraph, could easily be outlined and their projected area measured (Plate 1); there was a gap of

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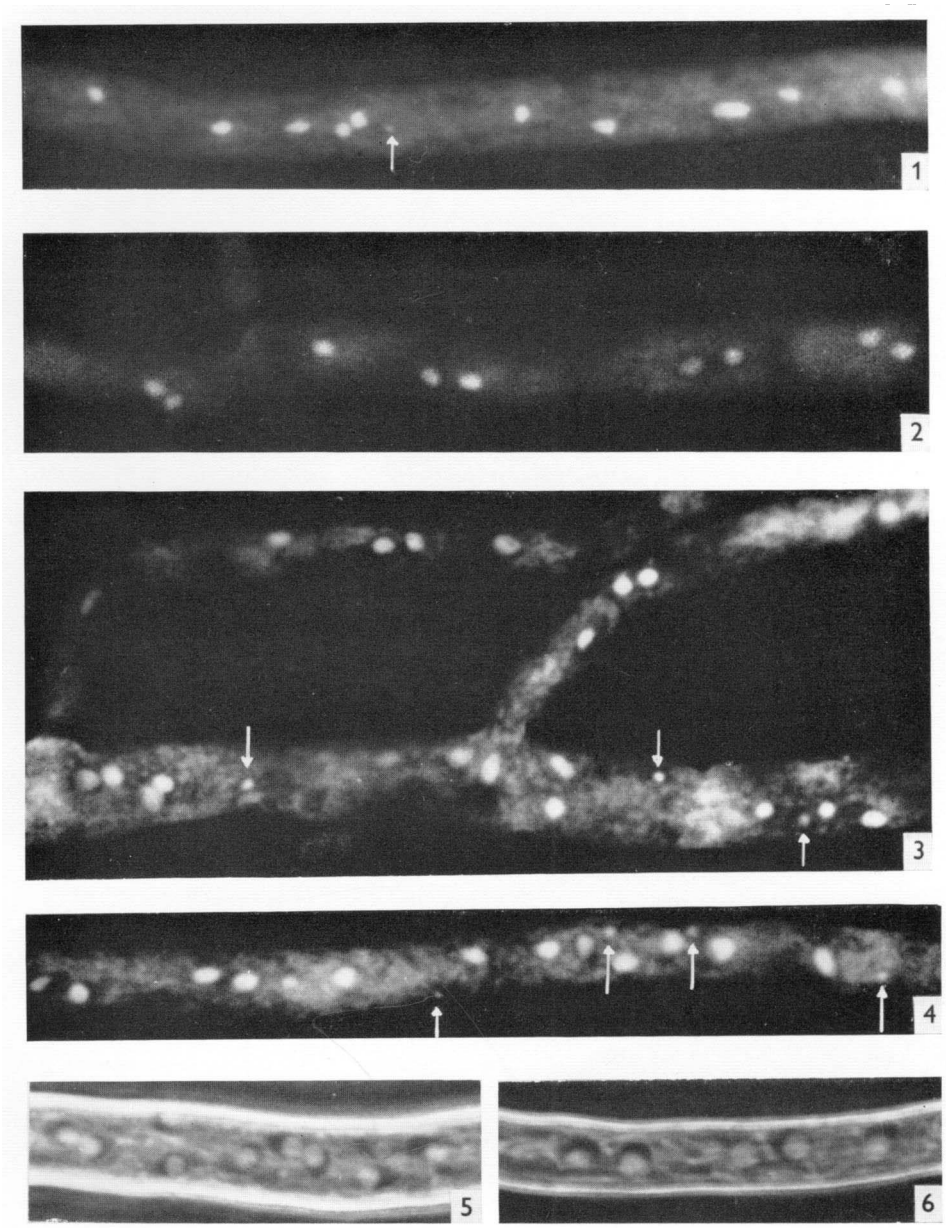

Figs. 1–4. Fluorescent staining. Figs. 5–6. Phase contrast. All × 1100.

Fig. 1. Fawn haploid on FPA 20. One micronucleus of the size typical in haploids is visible. Normal haploid nuclei are obviously smaller than diploid nuclei of the following figures.

Fig. 2. Diploid on CM 20.

Fig. 3. Diploid on FPA 20. Three micronuclei: note their size larger than the size of micronuclei in haploid.

Fig. 4. Diploid on FPA 40. Four micronuclei.

Figs. 5–6. Fawn haploid and diploid on CM 20. The nuclear size, although larger, compares with the nuclear size of fluorescent staining. No micronuclei visible: cytoplasmic organelles, most of them blurred because of their motion, should make the tracing of micronuclei very difficult in phase-contrast microscopy.

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(*Facing p.* 310)

https://doi.org/10.1017/S0016672300011897 Published online by Cambridge University Press

about $\frac{1}{2}\mu^2$ between the size of the micronuclei and that of the smallest normal nuclei in the haploid as well as in the diploid.

With respect to the number of normal nuclei per cell, both haploid and diploid show the same reaction to FPA: although the haploid always has more nuclei per cell than the diploid (between 1·8 and 2 times as many), the number after 20 h on FPA drops in both to more or less 65% of the number on CM, while after 40 h it goes up again to 83%. There seems therefore to be a direct effect of FPA on

Table 2. *Numbers of normal nuclei and micronuclei in haploid and diploid hyphal tip cells on CM and on FPA*

Strain	Treatment	No. of normal nuclei (mean ± S.E.)	No. of micronuclei (mean ± S.E.)	Ratio (%) micronuclei/ normals
Haploid	CM	262·4 ± 12·9	0·56 ± 0·13	0·21
	FPA 20	166·0 ± 6·4	1·40 ± 0·20	0·84
	FPA 40	218·5 ± 5·2	8·16 ± 0·36	3·73
Diploid	CM	132·8 ± 6·0	1·12 ± 0·21	0·84
	FPA 20	90·9 ± 3·1	9·76 ± 0·58	10·73
	FPA 40	111·3 ± 5·5	26·41 ± 1·18	23·72

the number of nuclei per cell: a noticeable reduction after 20 h, but also some adaptation of the mycelium to that substance after 40 h, reactions which are the same whatever the ploidy of the mycelium. A similar effect of FPA on the cellular area was detected, but as there was a correlation between both, it seemed better to confine the study to the number of nuclei. One can conclude that on FPA as well as on CM the number of nuclei per cell remains a criterion of the ploidy of a mycelium or of a part of it (Plate 1), and it is worth mentioning that, in parts of diploid cells on FPA 40, 1 μ^2 nuclei were several times met at the concentration characteristic of the nuclei in haploid cells.

Concomitant with its action on the number of normal nuclei, FPA brings about a change in the ratio micronuclei/normal nuclei per cell and this ratio, for the same treatment, is always lower in haploid than in diploid cells (Table 2). This action had been investigated by means of covariance analysis. Table 3.1 synthesizes the results of this analysis for differences between treatments. Three possible cases might be distinguished: either the regression of micronuclei on normal nuclei gives a number of straight lines with the same slope but different intercepts, or it has a single straight line as its theoretical counterpart, or both slopes and intercepts are the same. We simply observe that if the Fisher-Snedecor (Snedecor, 1934) F-test is not significant, the corresponding hypothesis should not be rejected. One notes that the haploid and the diploid behave differently; for the haploid, the first hypothesis (common slope of the regression lines) is not rejected, but the second and third hypotheses are; for the diploid, all hypotheses are significantly rejected, which means that the treatments have different slopes and intercepts.

Taking into account these results, one now tests whether there are significant differences in means among slides within treatments. The result (Table 3.2) shows

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that there are no significant differences at the 5% level except for haploid FPA40 and diploid CM20. The complete analysis for slopes and intercepts within treatments gave the following results: for haploid FPA40, the hypothesis of a single regression line for all the observations is not rejected [$F = 1{\cdot}70$; $F_{0{\cdot}95}(4,19) = 2{\cdot}90$], but for diploid CM20 there is more than one regression line [$F = 9{\cdot}36$; $F_{0{\cdot}95}(12,35) = 2{\cdot}03$] and the regression slopes are significantly different [$F = 10{\cdot}09$; $F_{0{\cdot}95}(6,35) = 2{\cdot}37$). These results will be interpreted below.

Table 3. *Analysis of covariance: difference between treatments (1) and difference between slides within treatments (2) for the number of micronuclei adjusted for the number of normal nuclei*

	Haploid		Diploid	
	D.F.	F ratio	D.F.	F ratio
(1) Difference between treatments				
Test for				
A single value of the slopes of the regression lines	2, 69	$\frac{0{\cdot}43}{1{\cdot}63} = 0{\cdot}27$	2, 141	$\frac{452{\cdot}81}{15{\cdot}02} = 30{\cdot}15^{***}$
A single common regression line	4, 69	$\frac{216{\cdot}88}{1{\cdot}63} = 133{\cdot}06^{***}$	4, 141	$\frac{4528{\cdot}79}{15{\cdot}02} = 301{\cdot}52^{***}$
Difference in intercepts	2, 71	$\frac{433{\cdot}32}{1{\cdot}60} = 270{\cdot}83^{***}$	2, 143	$\frac{8604{\cdot}76}{21{\cdot}14} = 407{\cdot}04^{***}$
(2) Difference within treatments				
Difference in intercepts				
CM 20	4, 19	$\frac{0{\cdot}14}{0{\cdot}51} = 0{\cdot}27$	6, 41	$\frac{5{\cdot}71}{1{\cdot}53} = 3{\cdot}73^{**}$
FPA 20	4, 19	$\frac{0{\cdot}54}{1{\cdot}15} = 0{\cdot}47$	6, 41	$\frac{4{\cdot}23}{14{\cdot}34} = 0{\cdot}29$
FPA 40	4, 19	$\frac{8{\cdot}24}{2{\cdot}39} = 3{\cdot}45^{*}$	6, 41	$\frac{58{\cdot}92}{25{\cdot}70} = 2{\cdot}29$

$^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

4. DISCUSSION

Dry weight of thin layer colonies, growth curves and nuclear content of haploid and diploid strains on CM and FPA all point to the same conclusion: the different action of FPA according to the ploidy of the strain.

For the haploids, the growth rate on CM goes up linearly from the time of germination until more or less 20 h later, when it reaches a maximum which is maintained from that moment, the linear increase being probably the result of a steady increase in length of the tip cells at each nuclear generation; on FPA, similar growth curves are obtained, but the maximum growth rate is lower than that which could have been expected from comparison with the haploids, and the plateau of the haploids is replaced by a linear descending slope, which goes down

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to what will be, after almost 40 h, the steady growth rate of the diploid on FPA, a radius increase of about one mm. per 24 h at 35 °C (Lhoas, 1967). Therefore, apart from the possible action of the amino acid analogue on the many processes which govern the growth rate, there seems to be a direct action affecting haploid and diploid nuclei in a different manner.

The cytological observation of the hyphal tip cells, responsible for the growth of the mycelia, confirms this conclusion. Haploidization itself of diploid nuclei is shown by the measure of the mean area of the nuclei and by the fact that the distributions of the nuclei of different size in diploids FPA 20 and FPA 40 are intermediate between the nuclear size distribution in the haploid and in the diploid on CM, the first one being more similar to the distribution in the diploid, the second one more like that of the haploid. FPA would therefore lead in the diploid to the formation of multiple monosomic and finally haploid nuclei.

This haploidization is the result of successive chromosome losses. Even in the haploid on CM there is always a certain proportion of nuclei, i.e. micronuclei, much smaller than that which can be considered as the normal nuclear size and this proportion increases with the length of the growth period on FPA; the same increase is found in the diploid on FPA, but is greater than in the haploid, although the proportion of micronuclei is already higher in the diploid than in the haploid. These micronuclei can only be considered as discarded nuclear fragments. One has therefore to accept an action of FPA on the nuclei leading to chromosome losses. That diploid nuclei discard more micronuclei than the haploids is easily explained first by the ploidy of the nuclei, secondly perhaps by the fact that monosomic nuclei can still discard other fragments, while nullisomic haploids cannot; this would be responsible for the clustering of small nuclei in the diploid on FPA, but would also explain the different action of FPA on the proportion of micronuclei.

This difference in action is evident in the adjustment of the number of micronuclei for the number of normal nuclei in the analysis of covariance. The simplest explanation of the difference lies in the fact that, after the loss of one or more chromosomes, the value of a nucleus in the cell is not the same whether this nucleus be haploid or diploid before the loss. In a haploid hypha, there can be two kinds of nuclei: normal ones, which can still lose one or more chromosomes, and nullisomics which cannot any longer; thus, in such a hypha, for each micronucleus—containing one or more chromosomes—there is one normal nucleus, or at least appearing to be normal, which does not play any role any more in the cell; in other words, there is a correlation between the number of micronuclei and of non-functional nuclei. In the diploid hypha on the contrary, some nuclei are normal, others are monosomic or multiple monosomic and these can still divide; in this case, there is no strict relation between the number of micronuclei and the number of abnormal ones. This would explain why, each time there is a difference in means of micronuclei—among slides or among treatments—there is only one regression line for means or one regression slope in the case of the haploid, but the regression slopes are different in the case of the diploid. This would at the same

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time explain the growth rates; in the haploid, a certain proportion of the nuclei newly formed at each division are useless for the cell because nullisomic; in the diploid, the amount of abnormal, but still functional, nuclei increases at each division, probably to reach a maximum corresponding to the minimum of the growth curve, but the exact relation between the number of aneuploid nuclei and the reduced growth rate still remains hypothetical.

One can conclude that, while FPA-induced haploidization seems, in some species (Day & Jones, 1966, 1968), to be the result of mutations to FPA-resistance and then of successive and naturally occurring chromosome losses (Käfer, 1961), in others it takes place in quite a different way: at least with the FPA concentration used to induce haploidization in *A. niger*, no evidence of FPA-resistance was ever noticed (Lhoas, 1967) and the same lack of resistance in FPA-segregants was demonstrated in *A. nidulans* (McCully & Forbes, 1965). There seems to be an action of FPA altogether different from the selection of resistant genotypes and the results presented here prove that, at least in *A. niger*, the first effect of FPA towards haploidization is the induction of chromosome losses.

SUMMARY

1. The comparison of the dry weight of thin layer haploid and diploid colonies of *A. niger* on complete medium and complete medium supplemented with *p*-fluorophenylalanine led to the conclusion that there is a difference in growth rates of hyphae under these different conditions.

2. The growth curves of the same strains on both media were established. On complete medium, haploids and diploid show a growth rate increasing linearly for about 20 h after germination and reaching a maximum which is then maintained. On *p*-fluorophenylalanine, the haploids show a similar curve, although the maximum growth rate reached and maintained is about half that on complete medium; for the diploid, however, the maximum is less than the corresponding one in the haploid and, once this maximum has been reached, the growth rate goes down linearly to a very low value which is then maintained.

3. The cytological study of the hyphal tip cell showed, in the presence of the amino acid analogue, a reduction of the mean size of the diploid nuclei together with an increase of the number of nuclear fragments. This explains the growth rates observed and is accepted as a confirmation that *p*-fluorophenylalanine, by its action on the mitosis, favours chromosome losses which lead finally to the production of haploid nuclei.

The author is grateful to Professor G. Pontecorvo for criticism and for providing the technical facilities required by this work. Sincere thanks are due to Dr A. J. Clutterbuck for advice and discussion during the course of this work.

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