GENETICS OF PHYTOPATHOGENIC FUNGI. IV. EXPERIMENTALLY INDUCED ALTERATIONS IN NUCLEAR RATIOS OF HETEROCARYONS OF FUSARIUM OXYSPORUM F. PISI[1,2]

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THE parasexual cycle in fungi is initiated by the formation of a heterocaryon. Consequently, genetically dissimiliar nuclei are intermingled within a common cytoplasm in which nuclear "fusion" may occur. When genotypically different haploid nuclei are involved, a heterozygous diploid nucleus results. During the multiplication of such nuclei, mitotic crossing over or haploidization or both may occur and spores may eventually be obtained which have either diploid nuclei homozygous for certain genetic markers or haploid nuclei containing new combinations of genetic markers. In either event, methods are now available for genetic studies using fungi lacking a perfect stage (PONTECORVO 1953).

BUXTON (1956) suggested that a genetic analysis of factors controlling or determining pathogenicity in the imperfect fungus *Fusarium oxysporum* f. *pisi* might be accomplished by using the parasexual cycle. TUVESON and GARBER (1959b) have shown that certain heterocaryons and diploid strains of this species yielded spores with nuclei carrying markers characteristic of only one of the component strains. These results were interpreted by assuming that selective forces favored either multiplication of one nuclear type or the incorporation of one nuclear type in the conidia. Genetics studies involving the parasexual cycle are based on the assumption that the inclusion of different nuclear types in spores is random (PONTECORVO and KAFER 1958). Situations in which certain nuclear types do not become incorporated in the spores do not lend themselves to a rational analysis.

This paper presents data indicating that it is possible to alter the presumptive selective force responsible for the anomalous situation in heterocaryons of *F. oxysporum* f. *pisi*.

MATERIALS AND METHODS

Origin of fungal stocks, cultural conditions, methods for obtaining and characterizing nutritionally deficient mutants, and for making heterocaryons have been described in detail by TUVESON and GARBER (1959a,b). Except for HET (heterocaryon) XVI, turbid spore-suspensions of each diauxotrophic strain were mixed

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and added to the surface of minimal medium plates. In preparing HET XVI, equal numbers of spores were added prior to plating the suspension.

In several experiments, the minimal medium was supplemented with the nutritional requirements of the component strain that was either not recovered or occurred in a minority of spores from the respective heterocaryons. Agar blocks containing mycelium were removed from the center of each heterocaryon actively growing on minimal medium and transferred to supplemented or unsupplemented minimal medium. Inoculated plates were incubated for 18–21 days at 26°C; spores were harvested to determine their nutritional requirements (TUVESON and GARBER 1959b).

The nutritional requirements of the eight diauxotrophic strains used in making heterocaryons were: try, his—tryptophane, histidine; nic, val—nicotinic acid, valine; lys, ade—lysine, adenine (or hypoxanthine); met, leu—methionine (or cysteine), leucine; bio, iv—biotin, isoleucine-valine; his, ade—histidine, adenine; glu, pab—glutamic acid, para-aminobenzoic acid; ino, arg—inositol, arginine.

RESULTS

The nutritional requirements of spores harvested from ten heterocaryons grown on minimal medium are presented in Table 1. The first five entries repre-

TABLE 1

Nutritional requirements of conidia harvested from heterocaryons of Fusarium oxysporum *f.* pisi *grown on minimal medium**

Heterocaryons	Component strains	Nutritional requirements
I	bio, iv–try, his	104 bio, iv
II	nic, val–his, ade	104 prototrophs
III	glu, pab–lys, ade	130 glu, pab
IV	met, leu–ino, arg	78 met, leu; 25 met; 1 leu
V	bio, iv–his, ade	104 bio, iv
VII	bio, iv–met, leu	145 bio, iv
IX	bio, iv–nic, val	98 bio, iv; 6 nic, val
XII	bio, iv–glu, pab	128 glu, pab; 1 bio, iv
XVI	his, ade–met, leu	192 his, ade
XVII	his, ade–glu, pab	78 his, ade

* HET I–V (TUVESON and GARBER 1959b).

sent data from previous experiments reported by TUVESON and GARBER (1959b) and are included for completeness. In two of the ten heterocaryons, spores with nuclei from one or the other of the component strains were recovered; in seven heterocaryons, spores had nuclei from only one component strain. In the former heterocaryons, one spore type greatly outnumbered the other one.

Spores from HET II yielded only prototrophic colonies. When this heterocaryon was stored at 5°C for six months, its spores again yielded only prototrophic colonies. TUVESON and GARBER (1959b) proposed that this anomalous heterocaryon was a diploid in which genetically unlike haploid nuclei had "fused"

during the period in which the heterocaryon was being formed. Also, it was postulated that the initial heterocaryotic mycelium which had been selected for transfer included a preponderance of diploid nuclei. To test this hypothesis, the component strains were again combined to form the heterocaryon by the same procedure. Conidia from the newly formed heterocaryon yielded 112 nic, val colonies and 103 his, ade colonies. When 1×10^5 spores from this heterocaryon were plated on minimal medium, two prototrophic colonies were obtained. These presumptive, diploids have not yet yielded spores with recombinant nuclei.

PITTENGER, KIMBALL and ATWOOD (1955) have shown that the nuclear ratio in heterocaryons of *Neurospora crassa* bears a simple relationship to the proportion of nuclei in the inoculum used to produce the heterocaryons. In HET XVI, the spores carried only the his, ade nucleus. This heterocaryon was synthesized using a spore suspension containing equal numbers of spores from each component strain. Spores harvested from this heterocaryon grown on minimal medium required histidine and adenine, indicating that the proportion of nuclear types in the inoculum did not bear any obvious relationship to the proportions obtained in conidia from the heterocaryon.

HET VII yielded only spores requiring biotin, isoleucine and valine (Table 1). It was assumed that the mycelium of this heterocaryon might include a preponderance of bio, iv nuclei needed to provide for the adequate synthesis of methionine and leucine required by nuclei of the other component strain. To test this hypothesis, the heterocaryon was grown on minimal medium supplemented with graded concentrations on methionine and leucine and the harvested spores were tested for their nutritional requirements (Table 2). The frequency of spores

TABLE 2

Nutritional requirements of conidia harvested from HET VII grown on supplemented minimal medium

Component strains	Concentration of supplements	Nutritional requirements
bio, iv– met, leu	Minimal control	104 bio, iv
	.1 mg/ml met & .2 mg/ml leu	74 bio, iv 56 met, leu
	.2 mg/ml met & .4 mg/ml leu	53 bio, iv 51 met, leu
	.3 mg/ml met & .6 mg/ml leu	27 bio, iv 75 met, leu
	.4 mg/ml met & .8 mg/ml leu	1 bio, iv 102 met, leu

met = methionine; leu = leucine.

with met, leu nuclei increased in proportion with the concentration of methionine and leucine. At the maximum concentration, spores with bio, iv nuclei had almost disappeared. These observations indicated that it was possible to alter the selective forces present in heterocaryons involving nutritionally deficient mutant strains by supplementing the minimal medium.

HET VII was grown on minimal medium supplemented with methionine or leucine to determine if both compounds were needed to alter the nuclear proportions. The concentrations of these amino acids were selected on the basis of the previous experiment so that approximately equal numbers of nuclear types could be expected. According to data in Table 3, leucine but not methionine was effective in altering nuclear ratios.

TABLE 3

Nutritional requirements of conidia harvested from HET VII grown on singly supplemented minimal medium

Component strains	Concentration of supplements	Nutritional requirements
bio, iv–	.2 mg/ml met	104 bio, iv
met, leu	.4 mg/ml leu	54 bio, iv 50 met, leu

met = methionine; leu = leucine.

Spores from HET V, grown on minimal medium, required biotin, isoleucine and valine; it was not possible to detect spores with the his, ade nucleus (Table 1). This heterocaryon was grown on minimal medium containing 0.3 mg of histidine and 0.015 mg of adenine per ml of medium for 18 days; the harvested spores yielded 75 bio, iv colonies and 55 his, ade colonies.

HET XVI included the same mutant met, leu strain involved in the previously discussed HET VII. Spores from HET XVI grown on minimal medium yielded only his, ade colonies. This heterocaryon was grown on minimal medium supplemented with the same concentrations of methionine and leucine used in the previous experiments with HET VII. The results are summarized in Table 4. At the lowest concentration, all the spores carried the formerly "minority" met, leu

TABLE 4

Nutritional requirements of conidia harvested from HET XVI grown on supplemented minimal medium

Component strains	Concentration of supplements	Nutritional requirements
his, ade– met, leu	Minimal control	104 his, ade
	.1 mg/ml met & .2 mg/ml leu	104 met, leu
	.2 mg/ml met & .4 mg/ml leu	104 met, leu
	.3 mg/ml met & .6 mg/ml leu	6 his, ade 98 met, leu
	.4 mg/ml met & .8 mg/ml leu	102 his, ade

met = methionine; leu = leucine.

nucleus. Not until three times the lowest concentration of each supplement had been added to the minimal medium was it possible to obtain spores with the his, ade nucleus. Finally, heterocaryons grown on the maximum concentration of supplements yielded only spores with the his, ade nucleus. In summary, the frequency of spores with the met, leu nucleus did not increase in proportion with increasing concentrations of methionine and leucine.

Attempts to alter nuclear ratios for spores harvested from HET III (glu, pab–lys, ade) grown on minimal medium supplemented with graded concentrations of lysine and adenine were unsuccessful. This heterocaryon continued to yield spores with the glu, pab nucleus even when the medium was supplemented with concentrations which were 15 times that required for optimal growth of the component strain requiring lysine and adenine. It is possible that this heterocaryon represents a type which is refractory to the manipulation of nuclear proportions by supplementing the medium.

DISCUSSION

Except for HET II, heterocaryons involving diauxotrophic mutant strains of *Fusarium oxysporum* f. *pisi* yielded spores bearing a nucleus from either only one or predominantly one of the component strains. It has been proposed that this situation reflected a very strong selection favoring the nuclei of one component strain in the spore-bearing mycelium of the heterocaryon. The factors determining the selected or "majority" nucleus are not immediately obvious. Rules cannot be formulated at this time to predict which nuclear component will be recovered in the spores of a particular heterocaryon. For example, HET XII yielded spores with the glu, pab nucleus; only one spore with the bio, iv nucleus was recovered. On the other hand, HET XVII yielded only spores with the his, ade nucleus and spores with the glu, pab nucleus were not discovered. In addition, notice that in HET V the bio, iv nucleus is recovered and that the his, ade nucleus was not observed. The nuclear component to be found in spores of heterocaryons of this species appears to be determined by the particular component strains used to make the heterocaryon.

According to PROUT, HUEBSCHMAN, LEVENE and RYAN (1953), the distribution of nuclei in heterocaryons of *Neurospora crassa* involving component strains with nutritional deficiencies seems to depend on the particular heterocaryon. By their method of plating conidia, it was possible to establish that both nuclear components were present in the mycelium of heterocaryons. Since heterocaryons of *F. oxysporum* f. *pisi* grown on minimal medium usually yielded spores with only one nuclear type, the proportion of nuclear types in the mycelium of heterocaryons of this species cannot be determined by plating conidia.

PITTENGER and ATWOOD (1956) have shown that the growth rate of Neurospora heterocaryons in which the initial nuclear input ratio was relatively extreme (500:1 to 50:1) was submaximal and that there was no adaptive change in the nuclear proportions. Growth-tube experiments with these heterocaryons indicated that the nuclear ratio was relatively constant during growth. When

changes in growth did occur, they were not adaptive and the nuclei in the majority increased in frequency. BEADLE and COONRADT (1944) had postulated that the ratio of the component nuclei in heterocaryons of Neurospora was a matter of chance, providing one nuclear type had no selective advantage over the other. Furthermore, they suggested that the selected nuclear proportion in heterocaryotic mycelium resulted in optimal growth but that this ratio may have any value within very wide limits when the component nuclei were relatively "recessive".

The initial nuclear input in preparing heterocaryons of *F. oxysporum* f. *pisi* did not appear to influence the nuclear ratio of the resulting spores. Furthermore, heterocaryons seemed to yield spores with only one nuclear type relatively early during the growth of the heterocaryon and continued to do so over an extended period of time. It is possible that the hyphae closely associated with the substrate are heterocaryotic and that the aerial hyhae from which spores are presumably derived are homocaryotic for the "majority" nucleus.

RYAN and LEDERBERG (1946) investigated reverse mutation and adaptation in a leucine-requiring mutant strain of *Neurospora crassa*. Reverse mutation to prototrophy resulted in a heterocaryon. When this heterocaryon was grown on minimal medium supplemented with leucine, hyphae which originally had included some leucine-independent nuclei yielded only spores requiring this amino acid. PITTENGER and ATWOOD (1956) found that supplementation was ineffective in altering nuclear ratios in heterocaryons involving mutant strains with nutritional deficiencies. Adding the nutritional requirements of the "majority" nucleus, however, increased the growth rate of those heterocaryons exhibiting submaximal growth without altering the nuclear ratio.

JINKS (1952) investigated a heterocaryon of *Penicillium cyclopium* involving two morphological mutant strains. The heterocaryon "dissociated" on minimal medium. When the heterocaryon was transferred from an apple pulp medium to one containing minimal medium supplemented with apple pulp, there was an alteration in the nuclear ratio. As the proportion of apple pulp increased, the frequency of the "minority" nuclei also increased. According to JINKS, the component nuclei differed in their rate of multiplication and the nuclear ratio was a function of the type of medium on which the heterocaryon was grown. BUXTON (1954) reported similar observations for a heterocaryon of *F. oxysporum* f. *gladioli* involving two morphological mutant strains. Media differing in carbon concentrations or in carbon nitrogen ratios exhibited a selective effect for one or the other component nuclei, that is, the nuclear ratio was altered according to the relative concentrations of carbon or nitrogen sources in the medium.

In certain cases, nuclear ratios in heterocaryons of *F. oxysporum* f. *pisi* were determined by selective forces that could be altered by supplementing the minimal medium with the nutritional requirements of the "minority" nucleus. It is not clear at this time how supplementation accomplished this alteration of nuclear ratios.

Although HET V and HET VII exhibited an altered nuclear ratio on supplemented minimal medium, HET III was unaffected. Although the "minority"

nuclei of HET VII required methionine and leucine, only one of the amino acids (leucine) was effective in altering nuclear ratios. Whereas graded concentrations of the requirements of the "minority" nucleus common to HET XVI and HET VII were related to the nuclear output in spores from these heterocaryons, similar concentrations yielded different results for these heterocaryons. These observations clearly indicate that it is not possible to predict the outcome of supplementing the minimal medium with respect to nuclear ratios in heterocaryons of *F. oxysporum* f. *pisi*.

The effect of supplementation on nuclear ratios may be a useful tool in obtaining diploid nuclei in heterocaryons of Fusarium and, perhaps, other imperfect fungal species. The probability of a "fusion" of nuclei of different component strains would be greatly enhanced if these nuclei occurred in approximately equal numbers.

SUMMARY

In two of ten heterocaryons of *Fusarium oxysporum* f. *pisi*, involving diauxotrophic mutant strains, spores carried nuclei from both of the component strains; in seven heterocaryons, spores carried nuclei from only one of the component strains. The tenth heterocaryon yielded prototrophic spores which were presumably heterozygous diploids. When this heterocaryon was made a second time, it yielded spores with one or the other component nuclei in approximately equal numbers.

Three heterocaryons were grown on minimal medium supplemented with graded concentrations of the compounds required by the "minority" nucleus. In one heterocaryon, there was a direct relation between the concentrations and the frequency of the "minority" nucleus; in the second heterocaryon, there was no direct relationship; and in the third heterocaryon, supplementation did not alter the nuclear ratio.

The behaviour of heterocaryons of *F. oxysporum* f. *pisi* and those of other fungal species, principally Neurospora, are discussed.

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