

PARAMEIOSIS IN THE ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae* (METSCH.) SOROKIN

E. Bagagli^{1,2}, M.C.C. Valadares^{1,3} and J.L. Azevedo¹

ABSTRACT

Variations in the parasexual cycle, especially in relation to diploid instability, have been described in several fungal species. The process has been designated "parameiosis"; it is characterized by the emergence, from heterokaryons, of haploid and diploid recombinants, as well as the typical diploids normally recovered in a parasexual cycle. In the present work the occurrence of a similar process in *Metarhizium anisopliae* has been investigated. Conidia from heterokaryons formed between well-marked mutant strains, when plated onto appropriate selective media, resulted in the recovery of at least three main groups of colonies. The first group consisted of very unstable diploids or hyperhaploids; these, on plating of their conidia, produced several types of recombinant haploids. The second group consisted of already stable haploid recombinants formed by the breakdown of diploid heterozygous nuclei before conidial formation; and a third group, heterokaryotic colonies, which segregate only parental types. Parameiosis has been found in several Deuteromycetes and may play an important role in increasing genetic variability in these fungi.

INTRODUCTION

The parasexual cycle, initially described for, and extensively studied in the fungus *Aspergillus nidulans* (Pontecorvo and Roper, 1952), has been observed in a

¹ Instituto de Genética, ESALQ-USP, Caixa Postal 83, 13400 Piracicaba, SP, Brasil. Send correspondence to J.L.A.

² Present address: Departamento de Microbiologia e Imunologia, UNESP, 18600 Botucatu, SP, Brasil.

³ CENARGEN/EMBRAPA, S.A.I.N., Parque Rural, 70000 Brasília, DF, Brasil.

large number of other species, mainly those of the imperfect group (Burnett, 1975; Azevedo, 1976; Azevedo, 1987). In addition to permitting basic genetic studies, and planned breeding of species of commercial interest, the parasexual cycle has been considered to be a possibly natural mechanism of recombination in imperfect fungi (Roper, 1985). Variations in the parasexual cycle, especially those related to diploid instability, have been detected in different species using both classical procedures (Tuveson and Coy, 1961; Fantini, 1962; Hastie, 1962, 1964; Das and Ilczuk, 1978; Bergeron and Messing-Al-Aidroos, 1982; Bonatelli Jr. *et al.*, 1983; Hastie and Heale, 1984) and by protoplast fusion (Ball and Hamlyn, 1982; Minut and Esser, 1983; Birkett and Hamlyn, 1985; Hamlyn *et al.*, 1985; Silveira and Azevedo, 1987). This type of instability is almost always associated with a high frequency of haploidization and mitotic crossing-over; rather than being an exception in the parasexual cycle, it probably reflects the existence of a further mechanism for the increase of genetic variability in fungi. The term "parameiosis" has been proposed by Bonatelli Jr. *et al.* (1983) to designate this process.

Evidence of high diploid instability in the entomopathogenic fungus *Metarhizium anisopliae*, initially observed by Bergeron and Messing-Al-Aidroos (1982), was also detected by Silveira and Azevedo (1987) who used protoplast fusion. The objective of the present study was to determine the occurrence of recombinant haploid conidia originating directly from heterokaryons in *M. anisopliae* and to discuss the possible biological significance of this process in fungi.

MATERIAL AND METHODS

Strains

Two wild-type strains of *M. anisopliae* var. *anisopliae*, denoted E6 and E9, were used. For the origin of these strains, see Rosato *et al.* (1981). Mutants of these strains were obtained by UV light treatment and selected by the filtration and enrichment technique described by Silveira and Azevedo (1984) for the same fungus. The mutant strains used in the present study are shown in Table I.

Culture media and growth conditions

Minimal medium (MM) and complete medium (CM) were as described by Pontecorvo *et al.* (1953). Incubation was at 28°C. When necessary, MM was supple-

mented with nutritional requirements at concentrations of 50 $\mu\text{g/ml}$ for amino acids, 25 $\mu\text{g/ml}$ for adenine, 0.25 $\mu\text{g/ml}$ for riboflavin and 0.2 $\mu\text{g/ml}$ for biotin.

Table I - Strains of *Metarhizium anisopliae*.

Strain no.	Original strain	Genetic markers
E6/1	E6	vio2,ade5,pro11
E6/2	E6	vio2,ade5,met17
E6/7	E6	vio9,met9,bio9
E6/8	E6	ylo8,pyr8,lys8
E9/11	E9	met1,lys1
E9/12	E9	met1,lys3
E9/19	E9	ylo1,leu6,rib8
E9/20	E9	ylo1,leu6,ade9

ade, *bio*, *leu*, *lys*, *met*, *pro*, *pyr*, and *rib* indicate nutritional requirements for adenine, biotin, leucine, lysine, methionine (or cysteine), proline, pyridoxine and riboflavin, respectively; *vio* and *ylo*, pale vinaceous and yellow colored conidia, respectively, as opposed to the wild type green.

Genetic techniques

Heterokaryons were obtained between strains carrying complementary genetic markers by the technique of Roper (1952), as modified by Messias and Azevedo (1980) for *M. anisopliae*, using liquid MM with 4% CM (v/v). Conidial suspensions from the heterokaryons were plated onto CM to estimate the total number of viable conidia, and onto MM and MM plus appropriate nutritional supplements to recover prototrophs (MM) and, in addition, auxotrophic recombinants (supplemented MM) directly from heterokaryons (Cf in Hamlyn *et al.*, 1985). Single spore colonies of these recombinants, purified in CM, were classified for conidial colour and nutritional requirements. When necessary, ploidy was determined by the benlate test (Upshall *et al.*, 1976), by DNA quantification using the diphenylamine method (Giles and Myers, 1965), and by conidial and nuclear size measurements with an ocular micrometer (Valadares, 1989).

RESULTS AND DISCUSSION

Table II gives the heterokaryons studied. Heterokaryons between strains of the same origin (E6 + E6 and E9 + E9) showed vigorous growth with conidia of wild type (green) colour; heterokaryons between strains of different origin (E6 + E9) generally showed less vigorous growth, with cases in which the conidial colour was similar to that of one of the parents. This confirmed data reported by Silveira and Azevedo (1987), who obtained heterokaryons between strains of the same origin relatively easily, but had to use protoplast fusion to obtain easy production of heterokaryons between strains of different origins.

Table II - Crosses performed, heterokaryon vigour and colour of conidia of heterokaryons.

Crosses	Heterokaryon vigour	Conidial colour
E6/1 + E6/8	+++	green
E6/2 + E6/8	+++	green
E6/1 + E6/7	+++	green
E9/11 + E9/20	+++	green
E9/12 + E9/20	+++	green
E6/1 + E9/20	+	pale vinaceous
E6/8 + E9/19	+	yellow
E6/8 + E9/20	++	green

+++ to + represent vigorous to relatively poor growth.

Conidia from the heterokaryons yielded, on MM, diploid, hyperhaploid and haploid prototrophic colonies; on selective, supplemented MM they gave, in addition, auxotrophic recombinants and heterokaryotic colonies in both conditions (Table III). The frequencies of selected colonies, mainly stable haploid recombinants, varied substantially from heterokaryon to heterokaryon, in the range of 0.1 to more than 1000 per 10^5 viable conidia. High proportions of these colonies had yellow or pale vinaceous conidia and were uniform in conidial color. This showed that diploid formation and recombination, with reduction to haploidy or hyperhaploidy, had occurred during heterokaryosis and prior to conidial formation.

Table III - Class and frequencies ($\times 10^{-5}$) of colonies obtained from conidia of heterokaryons plated onto Minimal medium (MM) and MM with nutritional requirements added (average of at least 4 plates).

Crosses	Media*	Diploid or hyperhaploid**	Stable haploid recombinants	Heterokaryotic colonies
E6/1 + E6/7	MM	+	3.0	3.5
	MMS	-	4.0	4.0
E6/1 + E6/8	MM	-	14.0	6.0
	MMS	+	21.0	7.0
E6/2 + E6/8	MM	+	7.0	21.0
	MMS	+	15.0	35.0
E9/11 + E9/20	MM	-	0.1	0.2
	MMS	-	2.7	6.3
E9/12 + E9/20	MM	-	0.1	0.3
	MMS	+	1.8	4.2
E6/1 + E9/20	MM	-	10.0	15.0
	MMS	-	11.0	11.0
E6/8 + E9/19	MM	-	0.3	2.7
	MMS	-	6.0	55.0
E6/8 + E9/20	MM	-	> 1000	0.0
	MMS	+	> 1000	0.0

* MM = Minimal medium; MMS = Supplemented Minimal media with two nutritional requirements, one from each parental strain. For instance, from the E6/1 + E6/8 heterokaryon, conidia were plated onto MM with adenine and pyridoxine; MM with adenine and lysine; MM with proline and pyridoxine and MM with proline and lysine. For the heterokaryon E6/1 + E9/20, where the two parents had non-allelic requirements for adenine, the supplemented media were: MM with proline and leucine; MM with adenine; MM with leucine and MM with proline.

**Occurrence (+) and non-occurrence (-); frequencies not estimated.

Single spore colonies derived from MM and from supplemented media and analyzed in terms of morphologic and nutritional requirements showed once again that it was possible to obtain recombinants directly from conidia of heterokaryons, as is shown for four analyzed heterokaryons (Table IV). Stability tests using benlate and length of conidia derived from these colonies, as well as the diameters of their nuclei and the determination of DNA amounts, demonstrated the conidia to be haploid. However, even though a large proportion of colonies grown on MM and supplemented media appeared to be prototrophic, some of them produced recombinant colonies when plated onto CM or were unstable and produced sectors. These colonies were probably derived from highly unstable diploid colonies or from aneuploid colonies. *M. anisopliae* diploids are highly unstable and undergo spontaneous haploidization in CM (Bergeron and Messing-Al-Aidroos, 1982). Indeed, the stability reported by Messias and Azevedo (1980) for diploids in medium containing benlate may have been due to the rapid derivation of recombinant haploids from the unstable diploid obtained. As also suggested by Bergeron and Messing-Al-Aidroos (1982), the initial data reported by Messias and Azevedo (1980) and Al-Aidroos (1980) showing no difference in size between possible diploid conidia and haploid conidia must have been due to the analysis of haploid conidia derived from these highly unstable diploids. When nuclei diameter and conidial length of the possible diploid colonies were measured, higher values were actually obtained than for control haploids. In some cases, intermediate values were obtained, suggesting the existence of hyperhaploid forms that may represent possible intermediates in the process leading to haploidization. Haploidization by non-disjunction with chromosome losses until the haploid state is reached has been extensively investigated by Kafer (1961) and has been proposed as the process through which mitotic haploidization is obtained in *A. nidulans*. If the same process is occurring in *M. anisopliae*, it seems to be much more rapid.

The number of linkage groups of *M. anisopliae* is small, probably not exceeding five (Bergeron and Messing-Al-Aidroos, 1982). In the crosses performed, parental strains, each with two auxotrophic and colour markers, were used. On the basis of the data obtained, it was difficult to establish recombination rates between markers because of the probable high frequency of mitotic crossing-over occurring in association with haploidization.

In fungi in general, sexual reproduction is relatively limited and asexual reproduction intense, with the production of large numbers of vegetative propagula over short life cycles (Alexopoulos and Mims, 1980). This characteristic may easily generate imbalance or even extinction of one of the mating-types in heterothallic fungi, leading to a decrease or a loss of ability to reproduce sexually (Thomas *et al.*, 1983). The parasexual cycle, with its stages of heterokaryosis, nuclear fusion,

Table IV - Colour and requirements of *M. anisopliae* haploid recombinant colonies derived from conidia of heterokaryons plated onto different selective media, as confirmed by auxanographic analysis*.

Heterokaryon	Selective medium	Type of recombinant obtained
E6/1 + E6/8	MM	wild-type ylo
	MM + ade + lys	wild-type ylo
	MM + ade + pyr	ylo, pyr pyr
E6/2 + E6/8	MM	ylo vio
	MM + ade + pyr	ylo, pyr ylo, ade
	MM + met + pyr	ylo
E6/1 + E9/20	MM	vio
	MM + ade	vio
	MM + leu	vio leu
	MM + pro	vio
	MM + pro + leu	wild-type leu
E6/8 + E9/20	MM	ylo
	MM + leu + lys	ylo, leu
	MM + leu + pyr	ylo, leu pir
	MM + lys + ade	wild-type ylo

* These colonies were stable when analyzed in the presence of benlate. DNA per conidium ranged from 17 to 21 x 10⁻¹⁴ g and did not differ from the E6 and E9 parental strains or from the auxotrophs analyzed. The nuclear diameter and conidial length of these recombinants were also similar to those of the parental strains.

diploidization and haploidization, is a possible natural mechanism for increased variability in different fungi, such as the genus *Verticillium* (Hastie and Heale, 1984). Even though among fungi, the diploid phase is quite common and easy to propagate, in general the haploid state is the more stable. The hypothesis of nutrient scarcity is an explanation for the maintenance of the haploid state in several groups of organisms (Lewis, 1985). This is of special relevance among fungi and is reflected not only in the degree of ploidy but also in the mycelial morphology that provides a high surface-volume ratio which must optimize nutritional and metabolic exchange with the environment. The high diploid instability with the possibility of crossing-over similar to that observed in parasexuality may then have been the result of an evolutive process that permitted fungi to have a simple and efficient system for increasing genetic variability.

It is interesting to note that the presence of a parasexual cycle has not been detected in *Neurospora crassa*, a heterothallic fungus with a very efficient sexual cycle. In turn, in *Aspergillus nidulans*, a homothallic fungus presenting a sexual cycle, a parasexual cycle does occur but there is no evidence of parameiosis. In contrast, parameiosis seems to occur in fungi with no sexual cycle such as *A. niger* (Bonatelli Jr. *et al.*, 1983), *M. anisopliae* (Bergeron and Messing-Al-Aidroos, 1982; Silveira and Azevedo, 1987), *Verticillium* (Hastie and Heale, 1984), *Beauveria bassiana* (Paccola-Meirelles and Azevedo, 1991) and *Trichoderma pseudokoningii* (Furlanetto, 1989). It could also be speculated that haploidization reflects the commitment of some diploid nuclei to a process which resembles meiosis but without the need of special sexual structures (Bonatelli Jr. and Azevedo, 1990).

By providing the derivation of recombinants at relatively high frequencies, parameiosis has proved to be an important process for obtaining new combinations between *M. anisopliae* strains with greater potential for the biological control of insects (Bagagli, 1987; Bagagli and Azevedo, 1987) and may also be quite useful for other species of economic interest that do not have a sexual phase.

ACKNOWLEDGMENTS

The work was partially supported by grants from EMBRAPA and FINEP. The first author acknowledges receipt of a FAPESP scholarhip.

Publication supported by FAPESP.

RESUMO

Variações no ciclo parasexual, especialmente relacionadas com instabilidade de diplóides já foram descritas em várias espécies de fungos. O processo foi chamado de "parameiose" e é caracterizado

pelo aparecimento, a partir de heterocários, de recombinantes haplóides e diplóides além dos diplóides heterozigotos normalmente encontrados. Na presente investigação um processo semelhante foi encontrado em *Metarhizium anisopliae*. Conídios obtidos de heterocários formados entre linhagens com marcadores genéticos apropriados foram semeados em meios seletivos e pelo menos três grupos de colônias foram obtidos. O primeiro deles consistiu de diplóides ou hiperhaplóides altamente instáveis que por sementeira de seus conídios produziram vários tipos de recombinantes haplóides. O segundo grupo era formado de recombinantes haplóides estáveis que devem ter-se originado de núcleos diplóides que sofreram um processo de haploidização dentro das hifas heterocarióticas antes de serem incorporadas aos conídios; e o terceiro grupo de colônias heterocarióticas as quais segregaram apenas os tipos parentais. A parameiose já foi encontrada em várias espécies de Deuteromicetos e deve ter um papel importante no aumento da variabilidade genética desses fungos.

REFERENCES

- Al-Aidroos, J. (1980). Demonstration of a parasexual cycle in the entomopathogenic fungus. *Metarhizium anisopliae*. *Can. J. Genet. Cytol.* 22: 309-314.
- Alexopoulos, C.J. and Mims, C.W. (1980). *Introductory mycology*. 3rd ed. John Wiley and Sons. New York.
- Azevedo, J.L. (1976). Variabilidade em fungos fitopatogênicos. *Summa Phytopathol.* 2: 3-15.
- Azevedo, J.L. (1987). Recombinação em fungos filamentosos. In: *Genética Molecular e de Microrganismos* (Costa, S.O.P., ed.). São Paulo, Brasil. Editora Manole Ltda., pp. 393-407.
- Ball, C. and Hamlyn, P.F. (1982). Genetic recombination studies with *Cephalosporium acremonium* related to the production of the industrially important antibiotic cephalosporin C. *Rev. Bras. Genet.* 5: 1-13.
- Bagagli, E. (1987). Parameiose em *Metarhizium anisopliae* (Metsch.) Sorokin. Masters Thesis, ESALQ-USP.
- Bagagli, E. and Azevedo, J.L. (1987). Parameiose e melhoramento genético do fungo *Metarhizium anisopliae* usado no controle biológico de insetos. *Congr. Latino-Americano Biotecnologia I*: 8, Tucuman, Argentina (Abstract).
- Bergeron, D. and Messing-Al-Aidroos, K. (1982). Haploidization analysis of heterozygous diploids of the entomogenous fungus *Metarhizium anisopliae*. *Can. J. Genet. Cytol.* 24: 643-651.
- BirKett, J.A. and Hamlyn, P.F. (1985). Protoplast fusion and genetic analysis in *Cephalosporium*. In: *Fungal protoplast applications in Biochemistry and Genetics* (Peberdy, J.F., Ferenczy, L. eds.). New York, Marcell Dekker, pp. 225-240.
- Bonatelli Jr, R. and Azevedo, J.L. (1990). Parameiosis: a system increasing variability in parasexual fungi. *4th International Mycological Congress, Regensburg* (Abstract).
- Bonatelli Jr., R., Azevedo, J.L. and Valent, G.U. (1983). Parasexuality in a citric acid producing strain of *Aspergillus niger*. *Rev. Bras. Genet.* 6: 399-405.

- Burnett, J.H. (1975). *Mycogenetics*. London, John Wiley and Sons.
- Das, A. and Ilkzuk, Z. (1978). Spontaneous segregation of a heterozygous diploid of *Aspergillus niger*. *Folia Microbiol.* 23: 326-365.
- Fantini, A.A. (1962). Genetics and antibiotic production of *Emericellopsis* species. *Genetics* 47: 161-177.
- Furlanetto, M.C. (1989). Recombinação genética e produção de celulases em *Trichoderma pseudokoningii* var. *rifai*. Masters Thesis, ESALQ-USP.
- Giles, K.W. and Myers, A. (1965). An improvement diphenylamine method for the estimation of deoxyribonucleic acid. *Nature* 206: 93.
- Hamlyn, P.F., Birkett, J.A., Peres, C. and Pederdy, J.F. (1985). Protoplast fusion as a tool for genetic analysis in *Cephalosporium acremonium*. *J. Gen. Microbiol.* 131: 2813-2823.
- Hastie, A.C. (1962). Genetic recombination in the hop-wilt fungus *Verticillium albo-atrum*. *J. Gen. Microbiol.* 27: 373-382.
- Hastie, A.C. (1964). The parasexual cycle in *Verticillium albo-atrum*. *Genet. Res.* 5: 305-315.
- Hastie, A.C. and Heale, J. (1984). Genetics of *Verticillium* *Phytopathologia. Mediterranea* 22: 130-162.
- Kafer, E. (1961). The process of spontaneous recombination in vegetative nuclei of *Aspergillus nidulans*. *Genetics* 46: 1581-1609.
- Lewis, Jr., W.M. (1985). Nutrient scarcity as an evolutionary cause of haploidy. *The Amer. Natur.* 125: 692-701.
- Messias, C.L. and Azevedo, J.L. (1980). Parasexuality in the Deuteromycete *Metarhizium anisopliae*. *Trans. Brit. Mycol. Soc.* 75: 473-477.
- Minut, W. and Esser, K. (1983). Intraspecific, interspecific and intergeneric recombination in beta-lactam producing fungi via protoplast fusion. *Eur. J. Appl. Microbiol. and Biotechnol.* 18: 38-46.
- Paccolla-Meirelles, L. and Azevedo, J.L. (1991). Parasexuality in *Beauveria bassiana*. *J. Inv. Pathol.* 57: 172-176.
- Pontecorvo, G. and Roper, J.A. (1952). Genetic analysis without sexual reproduction by means of polyploidy in *Aspergillus nidulans*. *J. Gen. Microbiol.* 6: vii.
- Pontecorvo, G., Roper, J.A., Hemmonms, L.M., MacDonald, K.D. and Bufton, A.W.J. (1953). The genetics of *Aspergillus nidulans*. *Adv. Genet.* 5: 141-238.
- Roper, J.A. (1952). Production of heterozygous diploids in filamentous fungi. *Experientia* 8: 14-15.
- Roper, J.A. (1985). Recombinação em fungos filamentosos. In: *Genética de microrganismos em Biotecnologia em Engenharia Genética*. (Azevedo, J.L., ed.). Piracicaba, Editorial Fealq, pp. 29-33.
- Rosato, Y.B., Messias, C.L. and Azevedo, J.L. (1981). Production of extracellular enzymes by isolates of *Metarhizium anisopliae*. *J. Inv. Pathol.* 38: 1-3.
- Silveira, W.D. and Azevedo, J.L. (1984). Isolation of auxotrophic mutants of *Metarhizium anisopliae* by the filtration enrichment technique. *Rev. Bras. Genet.* 7: 1-8.
- Silveira, W.D. and Azevedo, J.L. (1987). Protoplast fusion and genetic recombination in *Metarhizium anisopliae*. *Enz. Microb. Technol.* 9: 149-152.
- Thomas, D.S., Suryanarayana, K. and Manavathu, E.K. (1983). Asexual reproduction coupled with heterothallism: possible consequences from fungi. *J. Theor. Biol.* 105: 373-378.

- Tuveson, R.W. and Coy, D.O. (1961). Heterokaryosis and somatic recombination in *Cephalosporium mycophilum*. *Mycologia* 53: 244-253.
- Upshall, A., Giddings, B. and Mortimore, I.D. (1976). The use of benlate for distinguishing between haploid and diploid strains of *Aspergillus nidulans* and *Aspergillus terreus*. *J. Gen. Microbiol.* 100: 413-418.
- Valadares, M.C.C. (1989). Genética e produção de exoenzimas em linhagens de *Metarhizium anisopliae* (Metsh.) Sorokin. Masters thesis, ESALQ-USP.

(Received January 15, 1990)