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Parassexualidade em fungos filamentosos: mecanismo, ocorrência e aplicações em estudos de genotoxicidade

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração – Biologia Celular), da Universidade Estadual de Maringá para a obtenção do grau de Doutor em Ciências Biológicas.

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Ao meu esposo, Tonny Ranghel Colli, pelo seu amor, incentivo e compreensão durante todos esses anos.

Aos meus pais, Oscar e Maria Helena pelo apoio constante na minha vida

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APRESENTAÇÃO

Esta tese é composta por dois artigos científicos que estão apresentados de acordo com as normas estabelecidas pelas revistas a que foram submetidos:

- Parasexuality in filamentous fungi: mechanism, occurrence and applications (Mini Review) (a ser submetido à revista Genetics and Molecular Biology).

- Genotoxic evaluation of sodium nitroprusside in *Aspergillus nidulans* (aceito para publicação na revista Genetics and Molecular Biology em novembro de 2005).

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Parassexualidade em fungos filamentosos: mecanismo, ocorrência e aplicações em estudos de genotoxicidade

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RESUMO O ciclo parassexual inicia-se com a formação do heterocário, que consiste na existência de núcleos haplóides de composição genética distinta em um citoplasma comum. Um micélio heterocariótico é formado através da anastomose de hifas homocarióticas. O processo pode resultar na fusão de dois núcleos haplóides distintos, no interior das hifas heterocarióticas, originando núcleos diplóides heterozigotos que estão sujeitos a sucessivas divisões mitóticas. Durante essas divisões, dois processos podem ocorrer: não-disjunção cromossômica e o crossing-over mitótico. Em algumas espécies de fungos, os núcleos diplóides formados no interior das hifas heterocarióticas são altamente instáveis, sofrendo recombinação e haploidização antes da produção de conídios e permitindo a obtenção de haplóides recombinantes diretamente do micélio heterocariótico, sem a recuperação da fase diplóide. Este processo foi denominado ciclo parassexual com parameiose. A viabilidade do heterocário depende da constituição genética das linhagens em relação aos loci denominados *het* (incompatibilidade do heterocário) ou *vic* (incompatibilidade vegetativa). Linhagens que apresentam identidade alélica para os loci *het* ou *vic* são descritas como membros de um mesmo grupo de compatibilidade vegetativa (VCG). A recombinação mitótica associada ao ciclo parassexual pode ser induzida por agentes físicos e químicos que promovem lesões na molécula de DNA. Dentre estes podemos citar as radiações ionizantes e não-ionizantes, o nitroprussiato de sódio, os venenos de topoisomerase II, tais como doxorrubicina e etoposida, os inibidores da síntese de DNA, tais como danofloxacina, norfloxacina e 5-azacitidina, e os agentes que promovem alterações no ciclo celular tal como a vincristina. O potencial recombinagênico do nitroprussiato de sódio foi avaliado no presente trabalho utilizando-se duas linhagens diplóides do fungo *Aspergillus nidulans,* uma selvagem ($uvsH^{+}//uvsH^{+}$) e outra deficiente no reparo do DNA (*uvsH//uvsH*). O efeito recombinagênico da droga foi avaliado pela indução da homozigose de genes recessivos originalmente presentes em heterozigose. Os resultados demonstram que o nitroprussiato de sódio nas doses de 40 µM, 80 µM e 160 µM induz o crossing-over mitótico em ambas as linhagens estudadas. A droga também alterou o crescimento micelial da linhagem A757 e reduziu a produção de conídios, porém, não afetou o desenvolvimento temporal das células. O efeito recombinagênico do nitroprussiato de sódio reflete o efeito genotóxico da droga sobre o DNA e caracteriza-o como um agente promotor de neoplasias.

Parasexuality in filamentous fungi: mechanisms, occurrence and application in genotoxicity studies

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ABSTRAT The parasexual cycle starts with heterokaryon formation which consists of genetically distinct haploid nuclei in the same cytoplasm. The heterokaryon mycelium is formed by anastomosis of homokaryon hyphae. The fusion of two distinct haploid nuclei occurs within heterokaryon hyphae. Consequently a heterozygous diploid nucleous originates which undergoes successive mitotic divisions. Two processes, chromosome-non-disjunction and mitotic crossing-over, may occur during these division. In certain fungus species, diploid nuclei within the heterokaryotic hyphae are highly unstable and undergo recombination and haploidization prior to conidia production and obtain haploid recombinants directly from the heterokaryotic mycelium without recovering the diploid phase. The above process is called parasexual cycle with parameiosis. Heterokaryotic viability depends on the genetic constitution of strains with regard to *het* (heterokaryon incompatibility) or *vic* loci (vegetative incompatibility). Genetically identical strains for a set of *het* loci are able to constitute stable heterokaryons. They are described as forming part of the same vegetative compatible group (VCG). Mitotic recombination compled to parasexual cycle may be triggered by physical and chemical agents which form lesion in the DNA molecule. These agents may be ionizing and non-ionizing radiations, sodium nitroprusside, topoisomerase II poisons, such as doxorubicin and etoposide, inibitors of DNA synthesis, such as danofloxacin, norfloxacin and 5-azacitidine, agents that cause changes in the cell cycle, such as vincristine. Recombinagenic potential of sodium nitroprusside has been assessed using two diploid strains of the fungus *Aspergillus nidulans,* or rathen, a wild ($uvsH^{+}//uvsH^{+}$) and a DNA repair deficient strain (*uvsH//uvsH*). The drug´s recombinagenic effect was evaluated by the induction homozygosis of recessive gene which was originally in heterozygosis. Results show that sodium nitroprusside in the concentration of 40 μM, 80 μM and 160 μM was sufficient to induce mitotic crossing-over in the two above mentioned strains. The drug has also changed mycelium growth of strain A757 and reduced the conidia production but not affect the temporal development of cells.The recombinagenic effected of sodium nitroprusside reflect the genotoxic effect of the drug on DNA and characterizes it as a promotional agent of neoplasias.

Parasexuality in filamentous fungi:

mechanism, occurrence and applications

Mini Review

Parasexuality in filamentous fungi: mechanism, occurrence and applications

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Running Title: Parasexual cycle in fungi

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I. ABSTRACT

The process of parasexuality in fungi is presented, with special reference to the participation of the parasexual cycle in genetic variability derivation. The parasexual cycle starts with the formation of a single heterokaryon among strains of distinct genetic constitution belonging to the same vegetative compatibility group. Heterozygous diploid nuclei from heterokaryotic mycelium may follow three different pathways: a) propagate themselves by successive mitosis and produce new diploid recombinant or non-recombinant nuclei; b) produce unstable aneuploid nuclei untill their return to normal haploid state by means of random chromosome losses; c) undergo recombination and haploidization within the heterokaryotic hyphae and give rise to recombinant haploids. The development of a compatible heterokaryon is under strict genetic control where vegetative compatible strains are defined as members of the same vegetative compatibility group (VCG). The establishment of recombinants by mitotic crossing-over may be induced by physical and chemical agents, especially ionizing and non-ionizing radiations, DNA synthesis inhibitors and cell-cycle change-producing agents.

II. INTRODUCTION

Eumycota, or true fungi, are eukaryotic chemotrophic microorganisms, with only one nucleus, such as the yeasts, or multinucleated, such as the filamentous fungi. Most of Eumycota presents filamentous cells, named hyphae, that anastomose, in minor or larger degree, to form a dense network of filaments named mycelium. Hyphae anastomosis is an important stage for intra-hyphae communication, water and nutrient translocation between hyphae and maintenance of the colony's general homeostasis. Growth and hyphae directions are associated to a strict genetic control and diffusive chemical signals that regulate Spitzenkörper's complex process (Gregory 1984, Davidson et al. 1996, Glass et al. 2000).

Anastomosis may occur between hyphae of the same colony or between hyphae of genetically different fungi. The heterokaryon thus produced is not only important for the life cycle of many fungi, but it is the first step in the parasexual cycle (Xiang et al. 2002).

The heterokaryon's viability depends on the strains' genetic constitution with regard to loci called *het* (heterokaryon incompatibility) or *vic* (vegetative incompatibility). Although genetically identical strains for a set of *het* loci form stable heterokaryons, strains that differ in a single specific *het* or *vic* allele are unable to establish vegetatively stable heterokaryons. Vegetative incompatibility is characterized by hyphae compartimentation and cell lysing. Vegetative compatible strains, on the other hand, are members of the same vegetative compatibility group (VCG) (Saupe 2000).

Heterokaryon is an association of distinct haploid nuclei in the same cytoplasm. Certain haploid nuclei may fuse themselves and form diploid nuclei which may undergo successive mitotic divisions. Chromosome non-disjunction and mitotic crossing-over are two processes which may occur during such divisions (Kafer 1961).

Diploid nucleus is rarely reported in certain fungus species, probably because of the haploidization of the 2n nucleus still in the heterokaryotic hypha. If mitotic recombination occurs prior to the formation of conidia, the recombinant haploids may be directly isolated from the heterokaryon. The above process is called parasexual cycle with parameiosis (Bonatelli et al. 1983, Becker and Castro-Prado 2004, Becker and Castro-Prado 2005).

Physical and chemical agents, which cause lesions in the DNA molecule, may trigger mitotic recombination associated to the parasexual cycle. They are described as inductors of mitotic exchanges in eukaryotic cells. Ionizing and non-ionizing radiations, topoisomerase II poisons, such as doxorubicin and etoposide, inhibitors of DNA synthesis, such as norfloxacin and 5-azacitidin, and agents that trigger changes in the cell cycle, such as vincristine, are among these agents. (Abbadessa and Burdick 1963, Wood and Kafer 1969, Crebelli and Carere 1987, Franzoni et al. 1997, Franzoni and Castro-Prado 2000, Chiuchetta and Castro-Prado 2002a,b).

The mechanism of parasexuality in fungus and its importance as a natural tool for the production of genetic variability in perfect and imperfect fungi will be discussed in this current revision.

III. PARASEXUAL CYCLE AND GENETIC VARIABILITY

Genetic diversity in fungus is chiefly associated with processes of mutation and gene recombination. Whereas mutation consists of a single source of genetic material which is new to the species, the sexual cycle authorizes the recombination of hereditary characteristics by meiosis and gives rise to strains with distinct genotypes for different markers. On the other hand, in the case of asexually reproducing fungi the recombinant strains may even originate without meiosis, or rather, by parasexual cycle. Sexually reproducing fungi, such as *Emericella nidulans* (anamorph: *Aspergillus nidulans*) and *Magnoporthe grisea* (anamorph: *Pyricularia grisea*), reproduce by parasexual cycle too (Baron 1996, Adams et al. 1998, Souza et al. 2003).

Genetic variability caused by parasexual recombination was described for the first time by Pontecorvo et al. (1953) in researches on the ascomycete *A. nidulans*. Pontecorvo (1956) later suggested the term parasexuality to define processes producing recombinant strains without passing through the sexual cycle.

Caten (1981) listed some forty fungus species in which parasexual recombination has been found, albeit not necessarily the entire cycle. It seems that vegetative processes in diploid and somatic recombination are almost universal among filamentous fungi. There are exceptions as research with *Neurospora crassa* has shown (Roper 1966, Azevedo 1998) (Table 1).

The parasexual cycle starts with the formation of the heterokaryon which consists of distinct genetic composition nuclei in a common cytoplasm. Heterokaryotic mycelium is formed by the anastomosis of homokaryotic hyphae. The process may produce a fusion of two distinct haploid nuclei within the heterokaryotic hyphae and consequently the production of a heterozygotic diploid nucleus (Viaud et al. 1998). Diploid conidia show a frequency

range between $1/10^6$ and $1/10^7$ of haploid conidia in heterokaryons of *A. nidulans* (Azevedo 1998).

Heterozygous diploid nuclei may spontaneously give rise to recombinant diploid nuclei through mitotic crossing-over and/or to recombinant haploids by recombination and haploidization processes (Roper and Pritchard 1955, Pontocorvo and Kafer 1958, Kafer 1961).

Haploidization and randomized loss of one member of each chromosome pair through successive mitotic divisions could happen. Aneuploid nuclei with chromosome number different from the original 2n (2n + 1 or 2n - 1) are formed during haploidization by non-disjunction chromosome process. Through new mitotic non-disjunctions the aneuploid nuclei may return to their original haploid state carrying new gene combinations. This fact is due to the randomized segregation of the chromosomes or eventually to crossing-over prior to chromosome losses (Pacolla-Meirelles and Azevedo 1991, Griffiths et al. 2000, King and Insall 2003, Schoustra et al. 2004) (Figures 1 and 2).

Aneuploid strains in *A. nidulans*, macroscopically identified by their irregular border colonies and by a growth which is slower than normal haploid colonies, are mitotically unstable and spontaneously produce mitotic variants in their colonies (Pollard et al. 1968) (Figure 3).

Mitotic crossing-over was initially described in *Drosophila melanogaster* (Stern 1936). Since homologous chromatids may contact one another and establish mutual exchanges in their segments, they actually trigger the mitotic exchange process. This process is reported in heterozygous diploid cells during interphase in eukaryotes and provokes the manifestation of recessive genes previously masked by the dominant allele (Zimmermann 1971, 1992).

The segregation of sister chromatids in heterozygous diploid nuclei during mitosis is of paramount importance to detect mitotic crossing-over. There are three types of segregation. X-type segregation occurs when recombinant chromatids segregate towards opposite mitotic poles and furthers homozygosis of the genes in the distal position towards the exchange point. Segregation originates recombinant products by the origin of daughter cells which are phenotypically distinct from the original diploid. Recombinant chromatids in Z-type segregation segregate towards the same mitotic pole and originate daughter cells phenotypically identical to the original heterozygous diploid cells. Y-type segregation occurs when there is a fault in the segregation of daughter chromatids which segregate together towards the same daughter cell. The process, albeit rare, is equivalent to the reductional division in meiosis 1 (Figure 4) (Chua and Jinks-Robertson 1991, Beumer et al. 1998).

It seems that mitotic recombination in yeast begins in G1 phase of the cell cycle, prior to DNA replication (Wildenberg 1970). Resolution of mitotic crossing-over may occur before DNA duplication by cutting of strands which fail to participate in the mitotic crossing-over. The above-mentioned process gives rise to daughter cells which are phenotypically identical to the original one. The origin of homozygous cells for distal markers from the mitotic exchange point is only possible if G1-initiated crossing-over events are followed by DNA replication. The same cell pairs with recombinant or paternal phenotypes are thus originated. The latter is the result of mitotic crossing-over which has been started in G2 phase of the cell cycle (Figure 5). Mitotic crossing-over in yeasts may also occur in G2 phase when the alignment of chromosomes associated to mitotic recombination in G1 is maintained after DNA replication (Esposito 1978, Chua and Jinks-Robertson 1991).

Genetic variability resulting from the parasexual cycle has been largely studied in the context of several fungus species. These include ascomycetes, basideomycetes and deuteromycetes, with special reference to phytopathogenic fungi and to fungi with particular

interests to industry and medicine (Viaud et al. 1998, Castrillo et al. 2004). Genetic recombination without sexual reproduction is important the not only in genetic analysis but also in obtaining diploid strains and recombinant haploids.

In genetic analysis the parasexual cycle may be employed in the mapping of genes and in the detection of spontaneous and induced chromosome translocations. Preliminary studies on the phenotypical characterization of parasexual segregants in *A. nidulans* have identified chromosome translocations. When translocations are absent, the mitotic segregants present a linkage between markers within the same chromosome and an independent segregation between different chromosome markers. Translocation occurs when there is a complete linkage between markers known and mapped in distinct chromosomes (Kafer 1962; 1965). Similar analyses have made possible the mapping of new mutations (Mc Cully and Forbes 1965, Costa et al 2001, Marins and Castro-Prado 2005, Becker and Castro-Prado 2005).

IV. HYPHAE ANASTOMOSIS

Filamentous fungi grow like a filament-shaped multicellular and multinuclear network of cells called hyphae. The hyphae fusion in these fungi is named anastomosis and this phenomenon establishes an interconnected hyphae network known as mycelium (Gregory 1984). Mycelia are important in intra-hyphae communication and in the maintenance of the colony's homeostasis during its growth and reproduction. The growth of filamentous fungi consists of processes involving the extension of the hyphae tips, ramification and fusion. These processes undergo physiological and genetic controls which are still very scantily understood (Davidson et al. 1996).

Hyphal fusion is observed in the vegetative, asexual and sexual cycle of filamentous fungi. During the vegetative phase, fusion initially occours between spores germlings, and

later inside the mature vegetative colony. During the sexual phase, hiphae of opposite mating-types, in the case of heterothalic fungi, undergo anastomosis. The maintenance of the dikariotic phase, whitch is a prerequisite for kariogamy, also requises the fusion of hyphae and involves the formation of specialized structures such as the croziers, in the ascogenous hiphae of the ascomycetes. In the sexual cycle, anastomosis is also involved in the formation of multihyphal aggregates from which the multicellular fruitbodies are derived (Glass et al. 2004).

Hyphae anastomosis among vegetative colonies in filamentous fungi has been described by Buller (1933). In this process the hyphae establish contact with one another through a break in the cell wall and then by fusion of the plasmatic membranes. Fusion may occur not merely among hyphae of the same colony but among those of different ones. Heterokaryon is thus produced (Xiang et al. 2002). The heterokaryon is a pre-requisite for the occurrence of sexual and parasexual cycles in fungi and for the physiological cooperation in the exploitation of environmental resources. Vegetative incompatibility, however, may restrict heterokaryon formation (Glass and Kaneko 2003).

IV.I. PHYSIOLOGICAL CONTROL OF HYPHAE ANASTOMOSIS

Although hyphae anastomosis is a continuous event, three distinct physiological stages should be considered during fusion: pre-contact; post-contact and post-fusion stage (Figure 6) (Glass et al. 2000).

The pre-contact stage involves growth and direction of a new hyphae extremity that will establish contact with another hypha of the same or of a different colony. The extreme tip of a growing hypha contains a body termed the Spitzenkörper (apical body) which consists of a cluster of small, membrane-bound vesicles embedded in a meshwork of actin microfilaments. The Spitzenkörper is always present in growing tips, disappears when growth

stops, reappears when growth restarts, and its position within the apex changes when hyphae change direction (Bartnicki-Garcia et al 1995a, Glass et al. 2000, Glass et al. 2004).

The apical vesicles that make up the Spitzenkörper (Figure 7) are thought to be produced from Golgi bodies and then transported to the tip by elements of the cytoskeleton – probably the microtubules, actin microfilaments and motor proteins like myosin. The vesicles fuse with the plasma membrane at the tip, and release their contents. These contents almost certainly differ in the different types of vesicle, but are thought to include: enzymes involved in wall synthesis such as chitin synthase and glucan synthetase, enzymes involved in wall lysis, enzyme activators, and some preformed wall polymers such as mannoproteins, although most wall polymers are synthesised *in situ* at the tip (Howard 1981, Bartnicki-Garcia et al. 1995a, b).

Influx of Ca^{2+} ions in the cell produces the ion's high concentration in cytoplasm immediately below the plasmatic membrane of the hyphae's tips. The localization of exocytosis points is thus determined. The cell surface of the secretory vesicles during this process is fused to the cell's plasmatic membrane so that its contents could be discharged (Figure 8) (Jackson and Heath 1993, Regalado 1998).

The wall is thin and thought to be structurally weak at the extreme tip, enabling new wall materials to be inserted. So the structural integrity of the hyphal tip might depend on the "actin cap" - a meshwork of actin microfilaments. The wall is strengthened progressively behind the apex by cross-linking of wall polymers (Deacon 1997, Glass et al 2004).

Clearly, the process of tip growth is extremely complex, with many coordinated components. Any change in the balance of these components could alter the shape of the tip or the direction of growth (Deacon 1997).

Spitzenkörper process is extant in the post-fusion event. There is an initial adhesion between the hyphae's cell walls and then the tips cease to grow. Later, the cell wall is

destroyed, probably due to the release of hydrolytic enzymes in the contact point between the hyphae. The fusion of plasmatic membranes follows. The establishment of a new cell wall between hyphae involved in anastomosis is related to the release of the vesicles' contents that form the Spitzenkörper complex (Figure 6) (Hickey et al. 2002).

Spitzenkörper's process disappears in the post-contact event and a mixture of the hyphae's nuclear and cytoplasm contents occurs (Lópes-Franco and Bracker 1996).

IV. II. GENETIC CONTROL IN THE ANASTOMOSIS OF HYPHAE

The fusion of vegetative hyphae may be genetically controlled. Several hypotheses suggest that procedures involved in *Saccharomyces cerevisae*'s cell fusion may be present in other ascomycetes as well. Actually several genes involved in the cell fusion of *S. cerevisae* are well preserved in *N. crassa* (Galagan 2003).

Genes *PMR1, PEA2* and *AGA1*, important for the control of cell fusion in *S. cerevisae*, and preserved in *N. crassa.* The genes respectively codify the proteins that are active in the fusion of the plasmatic membrane, in the polarization of the cytoskeleton for the displacement of vesicle in Spitzenkörper process and in the synthesis and remodeling of the cell wall (Valtz and Herskowitz 1996, Heiman and Walte 2000).

On the other hand, other genes necessary for cell fusion in *S. cerevisae* do not seem to be well preserved in the genome of *N. crassa*, including *FUS1* and *FUS2*. The specific functions of the proteins produced by these genes are still not well defined even though their localization in the cell fusion zone suggests that these proteins participate in the process (Gammie et al. 1998, Glass et al. 2004).

V. HETEROKARYOSIS AND VEGETATIVE COMPATIBILITY GROUPS

Heterokaryon formation by the anastomosis of homokaryotic hyphae is an important process in the life cycle of several species of fungus. It is a pre-requisite element for sexual and parasexual reproduction processes (Jacobson et al. 1998; Xiang and Glass 2004). Heterokaryosis establishes the exchange of genetic and cytoplasmatic material among genetically distinct strains and the transfer of hypovirulent factors, such as (ds)RNAs (double strand RNA) (Van 1982, Nuss and Koltin 1990). It seems that in several fungi the heterokaryon formation process is limited by the existence of vegetative incompatibility system (Corner and Poulter 1989, Saupe 2000).

Sexual and vegetative heterokaryons may be completely distinct in some fungi species. Strains that are able to form a sexual heterokaryon may be incapable of forming a vegetative heterokaryon, or vice-versa. They are classified respectively as sexually or vegetatively compatible. The compatibility of the heterokaryon is controlled by a strict genetic system in which vegetatively compatible strains are included in the same vegetative compatibility group (VCG) (Figure 9). On the other hand, sexually compatible strains are controlled by one or more loci called mating-types which may have one or more alleles (Fincham et al. 1979, Glass and Kuldau 1992).

Allele and non-allele systems are the two types of the genetic system that control vegetative compatibility. In the former identical strains for a set of loci, called *het* (heterokaryon incompatibility) or *vic* (vegetative incompatibility) (Perkins 1988, Saupe 2000), are capable of forming a stable vegetative heterokaryon and share the same VCG. Alternatively the strains that differ in any loci (*het* or *vic*) are unable to form heterokaryons and are classified as vegetatively incompatible. In the non-allele system the allele of a certain

locus interacts with the allele of another locus and block heterokaryon formation (Chase and Ullrich 1990, Saupe 2000).

Vegetative incompatibility restricts nuclear transference and cytoplasmatic elements during the growth of the fungus. Although the fusion of hyphae (anastomosis) normally occurs among vegetatively incompatible nuclei bearers, the death of the heterokaryon occurs, after incompatible fusion, by mechanisms not yet understood (Leslie 1993).

Ten different *vic* loci have already been identified in *N. crassa*. They are mapped in five out of the seven chromosomes in the species. Two loci have multiple alleles (Leslie 1987). Eight *het* loci have been found in the fungus *A. nidulans* and multiple alleles are present in at least two loci (Croft and Dales 1984). Based on the segregation of several VCGs, ten *vic* loci were identified in *Fusarium moniliforme* in specific strain crossings (Puhalla and Spieth 1985).

Fungus *Cryphonectria parasitica*, a blight agent of the chestnut tree, produces cancer or lethal tumors in the tree bark and destroys the plant's vascular system. Hypovirulant strains of *C. parasitica* are infected with (ds)RNA virus which may be found in the cell cytoplasm. (ds)RNA may be transmitted from one strain to another through heterokaryosis which is the natural biological control process of chestnut tree blight. Nevertheless, the transmission of the hypovirulant phenotype may be restricted by the vegetative incompatibility system of *C. parasitica* involving at least six *vic* loci. Actually, strains that belong to different VCGs have already been isolated (Anagnostakis and Waggoner 1981, Anagnostakis 1982, Kuhlman and Bhattacharyya 1984, Bissegger et al. 1996).

Three methods are used to identify *vic* loci: a) direct evaluation of heterokaryon formation (normally by complementation between genetic markers); direct evaluation of incapability in heterokaryon formation (barrage formation); and c) strains which are

heterozygous for one or more *vic* loci and which present duplicate regions in the genome (Leslie 1993).

Direct heterokaryotic evaluation tests generally involve the formation of a stable prototrophic heterokaryon on selective medium, where not one of the two auxotrophic strains survives. Any genetic marker may, as a rule, be employed for the above evaluation. However, nutritional markers or resistance to fungicides markes, are preferred. This is due to the ease in characterizing the phenotype of the heterokaryon. Prototrophic heterokaryons shall be obtained only if involved strains are identified with regard to *het* or *vic* loci; otherwise, there will be no prototrophic growth (Figure 9) (Puhalla and Spieth 1985, Leslie 1993).

Barrage phenomenon to the development of heterokaryons occur in vegetatively incompatible strains through the antagonistic interaction of their hyphae. Restrictions are established in the contact region between the two colonies and the formation of a dark colored region, with dead cells, indicates its existence (Newhouse and MacDonald 1991).

vic loci may also be identified by obtaining strains which bear duplications of chromosome regions with vegetatively incompatible genes. It is the result of the construction of heterozygotic strains for a specific locus. Phenotypically aberrant, these strains have abnormal growth, color and morphology (Perkins 1975, Mylyk 1976).

The characterization of strains by determining vegetative compatible groups identifies clones from a common parent since strains of the same VCG are genetically similar. The method also identifies pathogenic strains and assists in gene mapping (Chaisrisook and Leslie 1990, Desjardins et al. 1992, Leslie 1993).

Since VCG application is related to the hypothesis that strains of the same pathogenic group integrate a specific VCG, its use as diagnostic tool is extremely valuable for phytopathologists (Phualla and Spieth 1985).

The characterization of phytopathogenic fungi in VCGs identifies distinct subgroups in fungus populations which were previously considered genotypically homogenous by molecular analyses (Busso and Castro-Prado, personal communication, Freeman et al. 2000).

VCGs were also employed to determine the clonal origin of *Fusarium moniliforme* strains, bearers of *pal1* and *fum1* mutations. Strains were separate in different VCGs and the hypothesis of clonal origin was discarded (Desjardins et al. 1992).

Vegetative incompatibility restriction, which hinders heterokaryon formation, may be artificially overcome by protoplast fusion technique, or rather, by cells whose cell wall has been enzymatically removed. This technique is an excellent method to obtain intra- and inter-species hybrids in several fungus species (Shubha 2004, Corner and Poulter 1989). Intra-species fusion of protoplasts is actually an efficient method to induce the parasexual cycle and obtain new strains by mitotic recombination (Anne 1983, Furlaneto and Pizzirani-Kleinr 1992).

VI. PARASEXUAL CYCLE WITH PARAMEIOSIS

In some fungus species, such as *Beauveria bassiana, Trichoderma pseudokoningii* and *Cephalosporium acremonium*, diploid nuclei formed within the heterokaryotic hyphae are highly unstable and undergo haploidization prior to conidia production. The process, called parasexual cycle with parameiosis, produces recombinants directly from the heterokaryotic mycelium without the recovery of the diploid phase (Bonatelli et al. 1983, Bagagli et al. 1991, Pacolla-Meirelles and Azevedo 1991, Ball and Jamlyn 1982). Although parameiosis has been initially described in imperfect fungi, the process has been recently reported in sexual-phase fungi, such as *Emericella nidulans* (anamorph: *Aspergillus nidulans*) and *Glomerella*

sublineolum (anamorph: *Colletotrichum sublineolum)* (Baptista et al. 2003, Souza-Paccola et al. 2003).

UV radiation sensitive mutants (*uvs*) have been used in *A. nidulans* in the formation of heterokaryons to isolate parameiosis segregants. Mutations that increase the intragenic mitotic recombination index (*uvsH* and *uvsF*) and mutations that alter chromosome segregation (*uvsC* and *uvsE*) are found among *uvs* mutations of the fungus. Homozygous and heterozygous heterokaryons in these mutations produced the two groups of mitotic segregants: stable haploids (parameiotic segregants) and aneuploids. Recombinant phenotypes of segregant haploids were characterized by analyses of their nutritional markers, resistance to acriflavin and conidia coloration. *uvs*-mutant heterokaryons produced 20.8% of parameiotic segregants. Control group, represented by heterokaryons formed with uvs+ strains, originated 4.55% of parameiotic segregants. All segregants classified were parameiotic are mitotically and meiotically stable (Baptista et al. 2003).

Genetic recombination without any sexual cycle occurs in the fungus *C. sublineolum*, the anthracnose agent in *Sorghum bicolor* (L.) Moench. Crossings between auxotrophic mutants and those resistant to Benomil and Cycloheximide confirm the occurrence of parameiosis in the species (Souza-Paccola et al. 2003).

Parameiosis may also be a tool to investigate the recombinagenic effect of DNA-affecting drugs (Becker and Castro-Prado 2004). Doxorubicin is an antineoplasic agent that may connect itself to DNA and inhibit the activities of enzyme topoisomerase II (Topo II). The drug has a recombinagenic effect and is capable of triggering secondary malignancies (Noviello et al. 1994, Chiuchetta and Castro-Prado 2002a). Heterokaryons formed between the *uvs* and *uvs+* strains of *A. nidulans* were inoculated in selective media with doxorubicin. Frequency of parameiotic segregants was significantly higher with doxorubicin than without it (Becker and Castro-Prado 2004).

Parameiosis, a natural recombination and genetic variability mechanism, may be used in genetic studies and in the production of strains of industrial interest (Bagagli et al. 1991).

VII. INDUCTION OF MITOTIC CROSSING-OVER

Mitotic crossing-over, one of the main DNA repair mechanism in eukaryotes, may be induced by physical and chemical agents (Haynes and Kunz 1981, Pires and Zucchi 1994). Defective yeast mutants in mitotic recombinants are unable to repair DNA damage especially double strand breaks (DSBs) (Resnick and Martin 1976, Ho 1975).

It is necessary that the cell have a second region in the genome homologous to the broken region so that DNA breaks may be repaired by the recombination process. Whereas DNA molecules in diploid cells are present in the form of homologous chromosomes, in haploid cells the homologue is provided by the sister chromatid which occurs in G2 phase of the cell cycle, posterior to DNA replication (Esposito 1978, Fabre 1978, Galli and Schiestl 1998).

During recombination repair the strands with initial cuttings produce a free tip 3' OH which invades the homologous region and forms D-loop (heteroduplex DNA region). The invading 3'OH is used as a primer and is extended by DNA synthesis. Since D-loop repairs the complementary strand, two Holliday junctions are produced which are solved by endonucleotidic cleavage giving rise to paternal or recombinant chromosomes (Figure 10) (Davies et al. 1975).

Ionizing and non-ionizing radiations are among the physical agents that trigger mitotic crossing-over. Similar to x-rays, ionizing radiations cause breaks in double DNA strands; similar to ultraviolet light (UV), non-ionizing radiations cause the formation of Cyclobutane

Pyrimidine Dimers (CPD) and 6-4 photoproducts (6-4PP) in the DNA molecule (Abbadessa and Burdick 1963, Wood and Kafer 1969).

X-ray-caused breaks may be repaired either by excision or by recombination (Haynes and Kuns 1981, Kupiec and Steinlauf 1997). Epistatic group genes *RAD 52* (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54* [*TID1*], *MRE11* [*RAD58*], and *XRS2*) in *S. cerevisae* are required for damage repairs induced by ionizing radiation. *RAD 52* mutants are faulty in mitotic recombination and, therefore, deficient in DSB repair. Several genes homologues to RAD 52 epistatic group genes and have been identified in other eukaryotes, mammals included. This fact shows the genes' high preservation in the recombinant repair pathway (Ho 1975, Davis and Symington 2004).

The association of UV light effect with mitotic recombination process has been originally described for yeasts by James (1955). Thenceforth several researches have shown the recombinagenic effect of this radiation in eukaryotic cells (Wood and Kafer 1969, Pires and Zucchi 1994). UV light-induced lesions (CPD and 6-4PP) produce serious distortions in the double helix and stimulate the repair mechanisms (Michell and Nairm 1989).

Faulty mutants for DNA repair in *A. nidulans* are divided into four epistatic groups: (1) Uvs F/H (*uvsF, uvsH* and *uvsJ*), (2) Uvs B (*uvsB* e *uvsD*), (3) UvsC (*uvsC, uvsE, musK, musL* and *musN*) and (4) UvsI (*uvsI*). The first mutant group presents normal levels of meiotic and high levels of mitotic recombinations. Mutants of the second group have changes in the cell cycle control and give rise to unstable segregants from diploid conidia. Mutants of UvsC epistatic group are faulty in recombinagenic repair and hyper-sensitive to DSB-inducing drugs. UvsI group mutants are faulty in mutagenic repair (Kafer and Mayor 1986, Goldman et al. 2002).

Diploid strains of *A. nidulans*, homozygous to *uvsH* mutation (UvsF/H group) have been used to evaluate the genotoxic activity of Cremofor EL, an organic solvent used in

chemotherapy protocols as a solvent of hydrophobic drugs and in food industries as emulsifier. Cremofor EL-treated diploids *uvsH//uvsH* show a significant increase in mitotic crossing-over frequency when compared to non-treated strains (Busso and Castro-Prado 2004).

Pires and Zucchi (1994) described a method to detect the genotoxic potential of chemical compounds by employing the parasexual cycle of ascomycete *A. nidulans*. It consists of obtaining prototrophic diploids, albeit heterozygous to conditional lethal mutations, by specifically using nutritional markers. Diploids are cultivated in a Minimum Medium supplemented with the chemical agent under analysis. Minimum Medium is made up of mineral salts, sodium nitrate and glucose so that only the growth of prototrophic strains occurs.

During culture diploid nuclei undergo successive mitosis and originate new diploid nuclei. In their turn, the latter may be heterozygous (+/- or -/+) or homozygous (+/+) for certain genetic markers. However, homozygous nuclei for recessive genes (-/-) are unable to develop in MM.

Somatic instability of the *A. nidulans*'s diploid nuclei is generally associated with the loss of genetic material during haploidization or with mitotic crossing-over (Gabrielli and Azevedo 1980, Geogpoulos et al. 1976, Parag and Roper 1975). Instability is shown by the origin of mitotic sections (or segregants) in the colonies of diploid strains. Mitotic segregants may be haploid, aneuploid or diploid, and present auxotrophy (Figures 2 and 3) (Pontecorvo and Roper 1953, Pontecorvo and Kafer 1958).

In genotoxicity testing, prototrophic diploid segregants obtained in the presence of the compound analyzed are purified in MM and haploidized in enriched culture medium (Complete Medium, Van de Vate and Jansen 1978). The ensuing haploids are analyzed phenotypically with regard to their nutritional markers. Homozygotization Index (HI) for each

analyzed marker is the quotient between the number of prototrophic and that of auxotrophic segregants. If the tested compound induces crossing-over in the original diploid, HI ≥ 2.0 is expected. On the other hand, if compound fails to induce mitotic recombination, HI rate will be lower than 2.0 (Figure 11).

Compounds that alter the DNA duplication and transcription processes, such as doxorubicin and etoposide, stimulate mitotic crossing-over in eukaryotic cells (Chiuchetta and Castro-Prado 2002a). These compounds are lethal to topoisomerase II (Topo II) since they link themselves to the DNA molecule and inhibit the catalytic activity of enzyme Topo II, which has the important function of maintaining DNA topology (Crebelli and Carere 1987, Boege 1996, Becker et al. 2003)

Compounds that induce cell cycle changes, such as antineoplasic vincristine, may also induce the somatic recombination process (Chiuchetta and Castro-Prado 2002b). Vincristine is an alkaloid that specifically binds itself to tubuline while damaging the mitotic apparatus by microtubule depolymerization and triggering cell cycle arrest (Blajeski et al. 2002, Holgersson et al. 2005).

5-azacitidine (5-AC) is a cytidine analogue with a nitrogen atom in position 5 of the pyrimidine ring. When incorporated to DNA, as a substitute for cytosine, this agent inhibits the activity of metyltransferase enzyme and causes hypometylation of the DNA molecule. The activation of mute genes by metylation of bases or by chromatin condensation may result (Rizwana and Hahn 1999, Haaf 1995, Barret 1993, Ramel et al. 1996).

The recombinagenic effect of 5-AC has been shown in *A. nidulans* by using a heterozygous diploid strain for nutritional, conidia coloration, and resistance to Acriflavin markers. Mitotic segregants were isolated from the 5-AC-treated diploid and its phenotypes analyzed. 5-AC showed to be effective in inducing mitotic crossing-over in some linkage intervals of chromosomes I and II of the diploid strain (Franzoni and Castro-Prado 2000).

Multiple genetic alterations, such as point mutations, chromosomal translocations and loss of heterozygosity are processes involved in the cancer development. In heterozygous cells for a tumor suppressor gene, the loss of the heterozygous condition may be the result of a mitotic crossing-over event that will originate clones of homozygous cells for the non-functional allele (Barrett 1993, Ramel et al. 1996). Thus studies have been developed to identify the genotoxicity effect of chemical substances and the parasexual cycle of *A. nidulans* represents a valuable tool in the identification of recombinogenic agents.

VIII. FUTURE PROSPECTS

Parasexual cycle been demonstrated in a great number of fungi incluind those of industrial importance, e.g. *A. nidulans*, entomopathogenic fungi, e.g. *M. anisopliae,* plant pathogenic fungi, e.g. *V. dohliae* and fungi of medical interest, e.g. *A .fumigatus.*

Parasexual recombination has been extensively used in genetic analysis of sexual and asexual fungi. Parasexual changes in genetic information among strains eliminate deleterious mutations and trigger genome reconstitution and the generation of new genotype variants.

Specific genotype diploids may be selected from heterokaryons that result from the randomized fusion of vegetative nuclei. These diploids may identify the interactions between allele pairs of the same gene or between mutation pairs in distinct loci.

Mitotic instability extent in diploid strains of perfect and imperfect fungi, such as *M. grisea* and *Verticillium albo-atrum*, support the hypothesis that the parasexual cycle is an important source of genetic variability. Direct evidences of parasexual exchanges extant in nature may be found in molecular studies the genotype structure of populations.

Recombinant haploids obtained by the parasexual cycle give information about markers from diferent chromosomes (inter-chromosome recombinants from independent assortment of

chromosomes) or about gene order on the chromosome with regard to the centromere (intra-chromosome recombinants from crossing-over) after the haploidization of a diploid nucleus. Parasexual recombination may thus be used in the mitotic mapping of mutations, chromosome aberrations and translocations. Gene alignment in the chromosomes produces a genetic map which serves as a basis for chromosome mapping by pulsed-field gel electrophoresis.

When molecular markers are actually employed to monitor parasexual recombination *in vitro* or in natural fungus populations, new knowledge on the mechanism and on the evolution meaning of parasexuality may be obtained.

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Table 1. Fungus species whose parasexual cycle has already been described (According to Azevedo, 1998).

Ascochya imperfecta	*Metarhizium anisopliae*
Aspergillus amstelodami	*Microsporum gypsium*
Aspergillus flavus	*Penicillium chrysogenum*
Aspergillus fumigatus	*Pencillium digitatum*
Aspergillus nidulans	*Penicillium expansum*
Aspergillus niger	*Penicillium italicum*
Aspergillus oryzae	*Phycomyces blakesleanus*
Aspergillus rugulosus	*Phymatotrichum omnivorum*
Aspergillus sojae	*Phytophthora infestans*
Beauveria bassiana	*Piricularia oryzae*
Cephalosporium acremonium	*Puccinia graminis tritici*
Cephalosporium mycophylum	*Saccharomyces cerevisae*
Cochiobolus sativus	*Schizaphyllum commune*
Coprinus fimetarius	*Trichoderma pseudokoningii*
Coprinus logopus	*Ustilago hordei*
Dyctyostelium discoidem (Mixomycete)	*Ustilago maydis*
Fusarium oxysporum f. sp. Callistephi	*Ustilago violácea*
Fusaruim oxysporum f. sp. Cubene	*Verticillium albo-atrum*
Fusarium oxysporum f. sp. Pisi	*Verticillium dahlias var. longisporum*

X. LEGENDS OF ILLUSTRATIONS

Figure 1: Schematic representation of normal chromosome segregation in mitosis and of chromosome segregation in mitotic non-disjunction (Modified from Griffits et al. 2002).

Figure 2: Diploid strain of fungus *Aspergillus nidulans* reverting to haploid nuclei (arrow) by haploidization.

Figure 3: (a): Aneuploid colonies of fungus *A. nidulans* macroscopically distinguishable by the production of haploid sections (arrows) by loss of a one of the homologues chromosomes. (b) and (c): Aneuploid colonies of fungus *A. nidulans* macroscopically distinguishable by irregular borders. (d): Haploid colonies of fungus *A. nidulans* without sections.

Figure 4: Schematic representation of sister chromatid segregation during mitosis (modified from Beuner et al. 1998).

Figure 5: Crossing-over resolution starting at G1 phase of the cell cycle prior to (a) or posterior to (b) DNA replication originates homozygous (c) or heterozygous (d) daughter cells for distal genes at the exchange site (modified from Chua and Jinks-Robertson 1991).

Figure 6: (a): Pre-contact, white points show Spitzenkörper's process at the growing hyphae's tips. (b): Post-fusion, Spitzenkörper's process persists and adhesion occurs between the cell walls of hyphae involved in anastomosis. Bar scale = 10µm (from Glass et al. 2004).

Figure 7. Simplified model of apical growth of fungal hyphae. G= Golgi, V= vesicle, M= microtubule. (from Deacon 1997)

Figure 8: (a): *S. cerevisae*: Sector vesicles directed by microfilaments of exocytosis. (b): Fungus hyphae: Sector vesicles, directed towards hyphae's tips by Spitzenkörper's process, undergo exocytosis. (c): Trajectory of Spitzenkörper's process which determine the direction of hyphae's growth. (d): Cytoplasmatic gradients of ions Ca^{++} or protons, pinpointing the site of exocytosis of vesicles (modified from Harold, 2002).

Figure 9**:** Heterokaryon formation between vegetatively compatible strains of *A. nidulans* (a) and *F. graminearun* (b) in selective medium. P_1 to P_5 = parental strains; H = heterokaryon.

Figure 10: DSB repair model. 3`OH tip invades homologous duplex and acts as primer for DNA synthesis. The two Holliday junctions are solved by endonucleotidic cleavage and give rise to crossing-over and non-crossing-over products.

Figure 11: Origin of heterozygous (-/+ and +/-) and homozygous (+/+) diploid strains after mitotic crossing-over between gene *paba* and centromere. * Homozygous diploids (-/-) are not selected in MM (modified from Pires and Zucchi 1994).

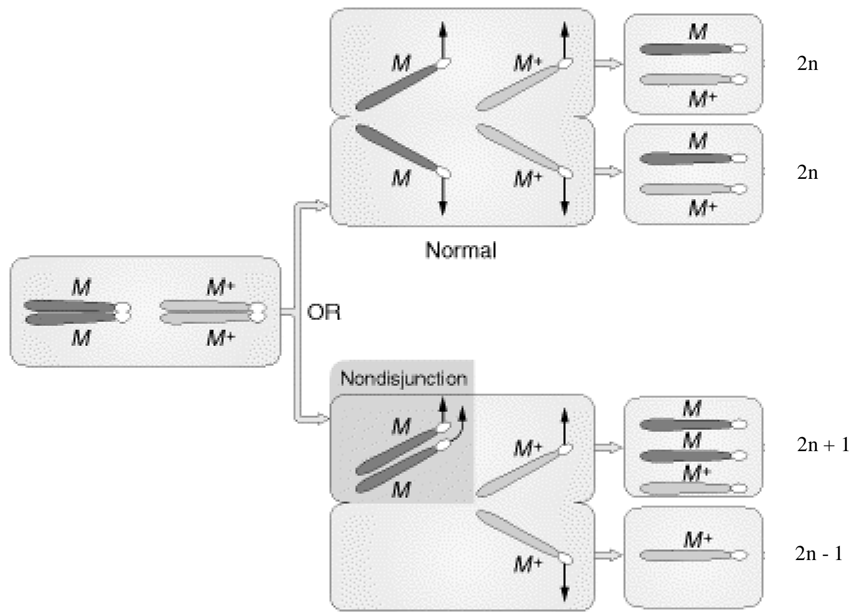


Figure 1

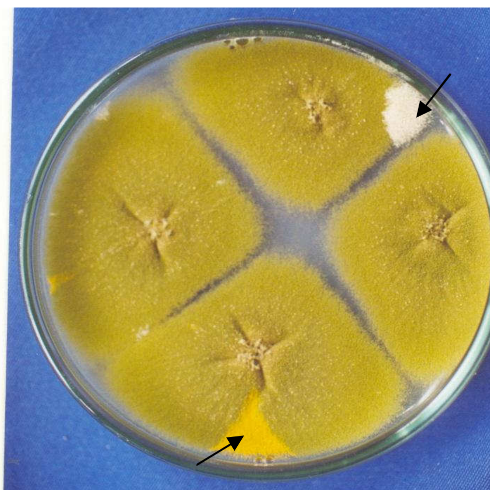


Figure 2

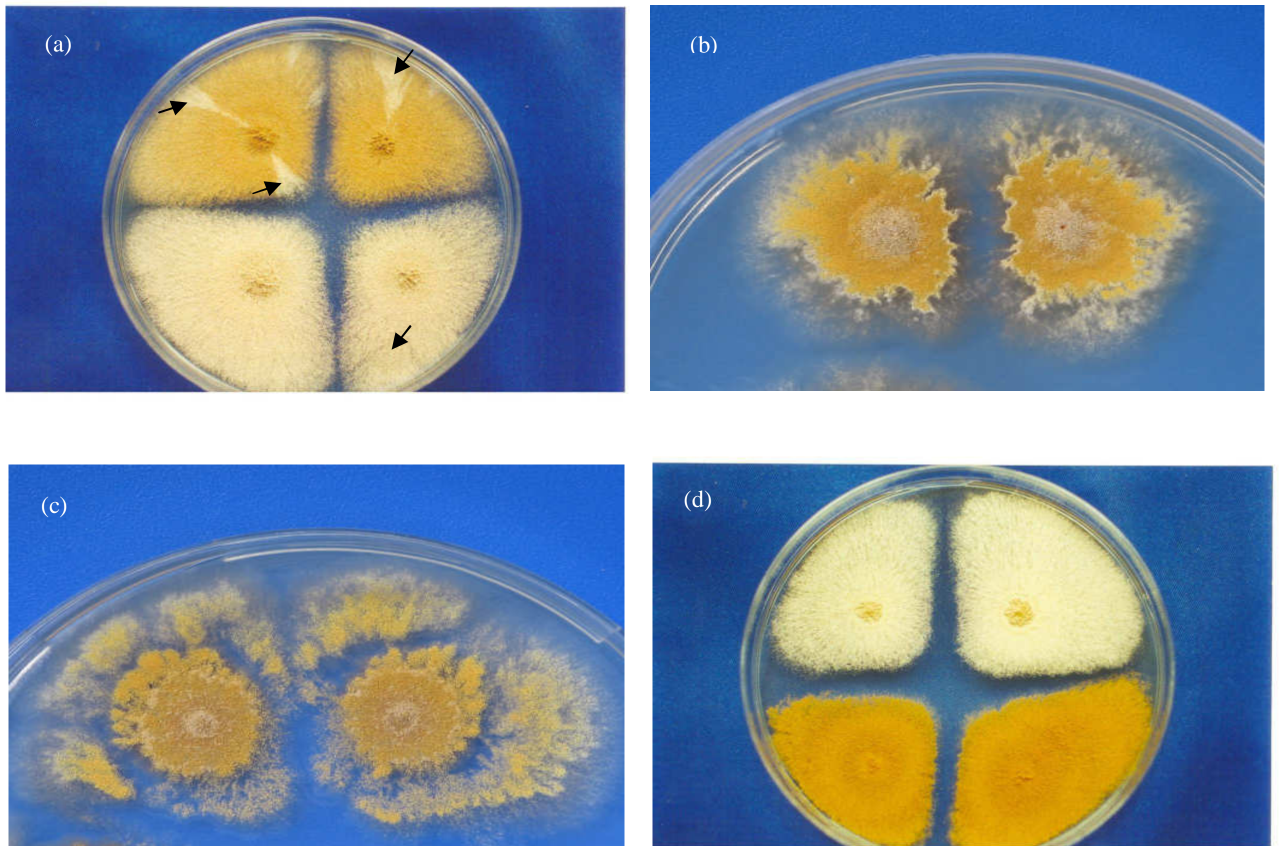


Figure 3

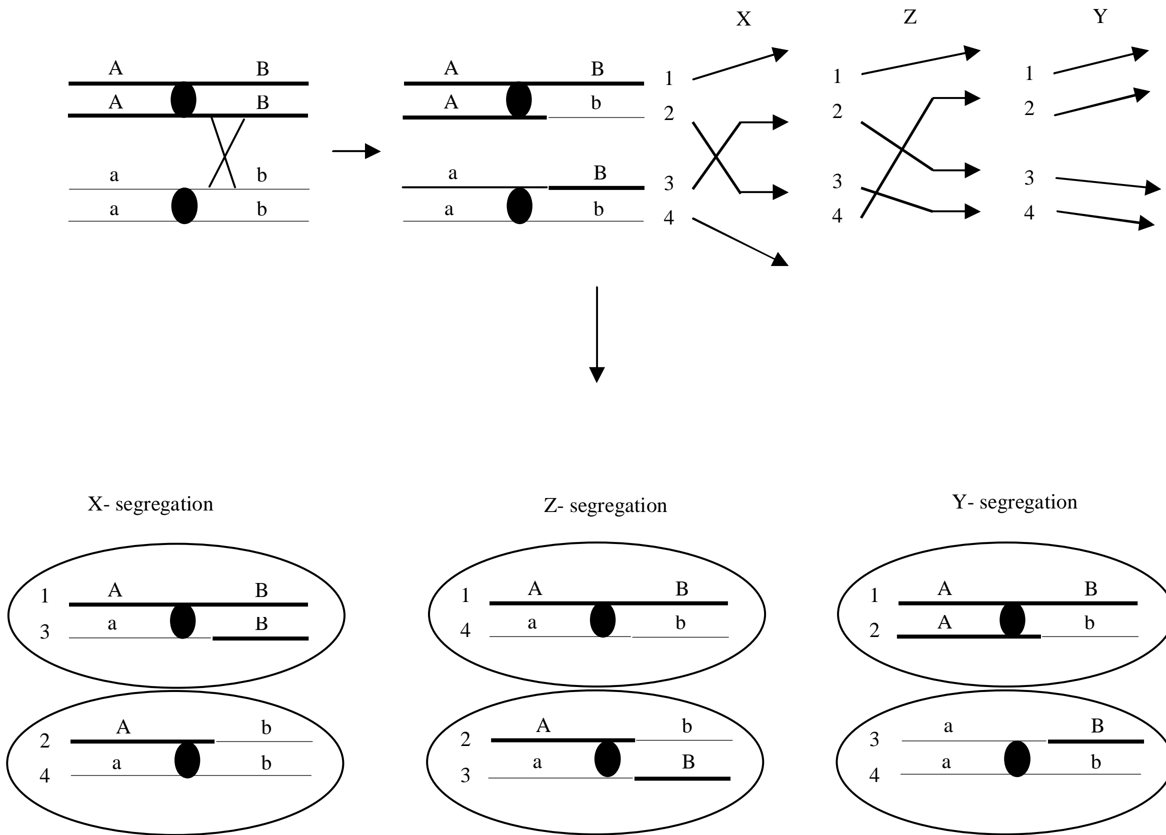


Figure 4

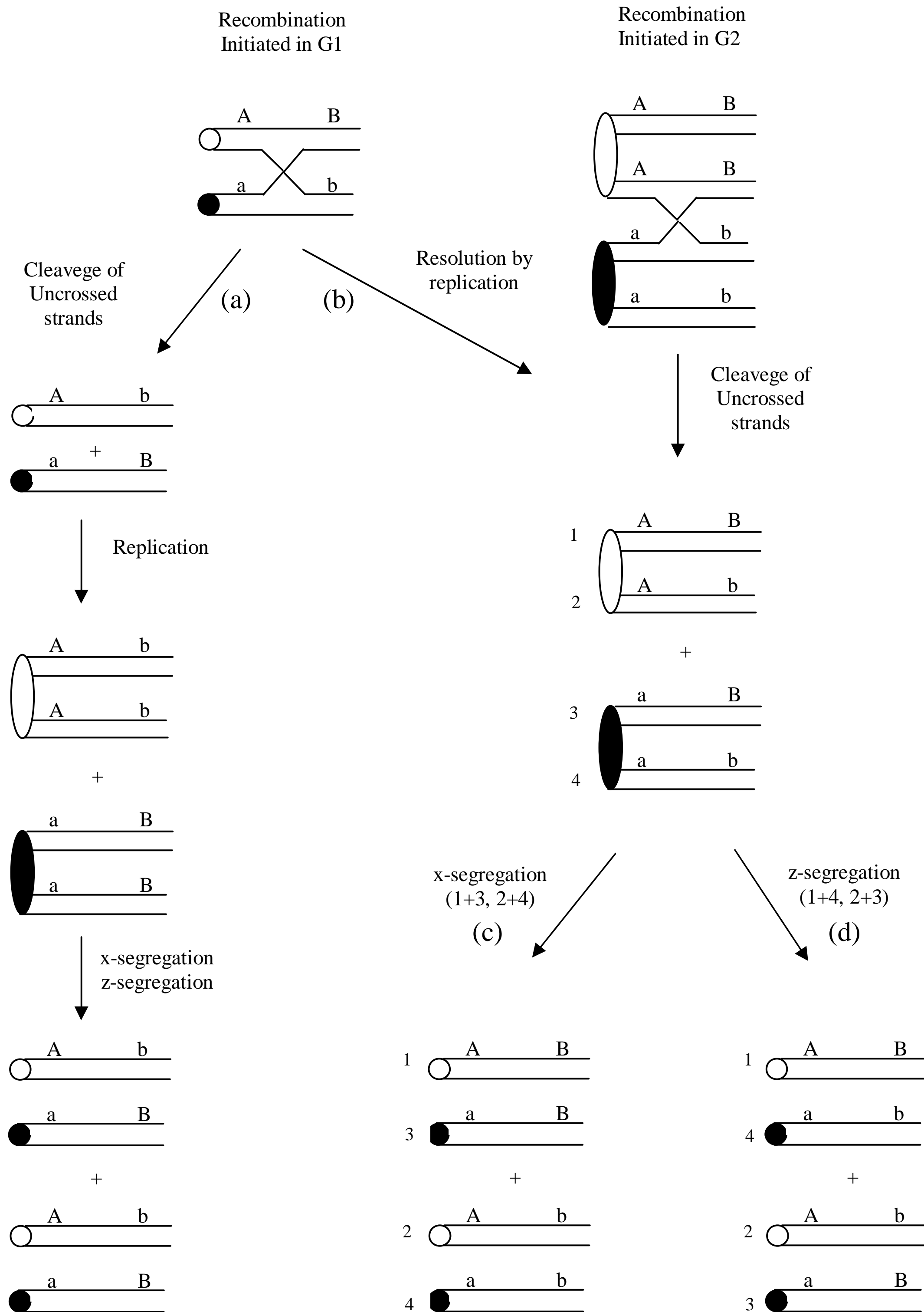


Figure 5

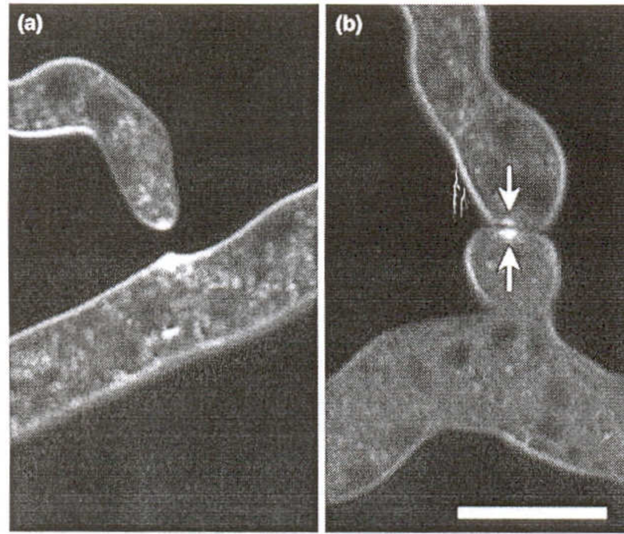


Figure 6

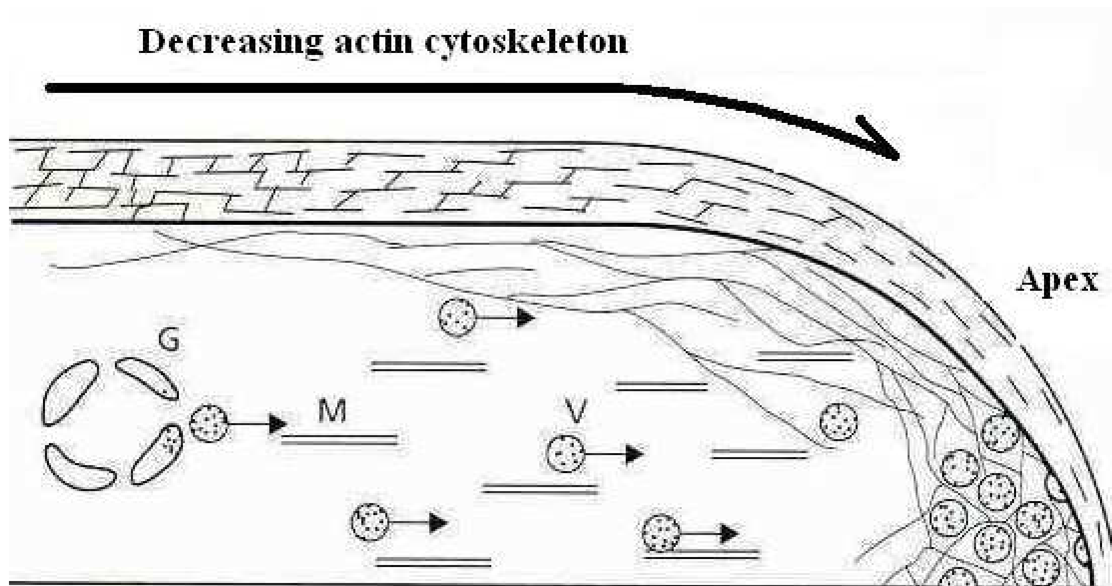


Figure 7

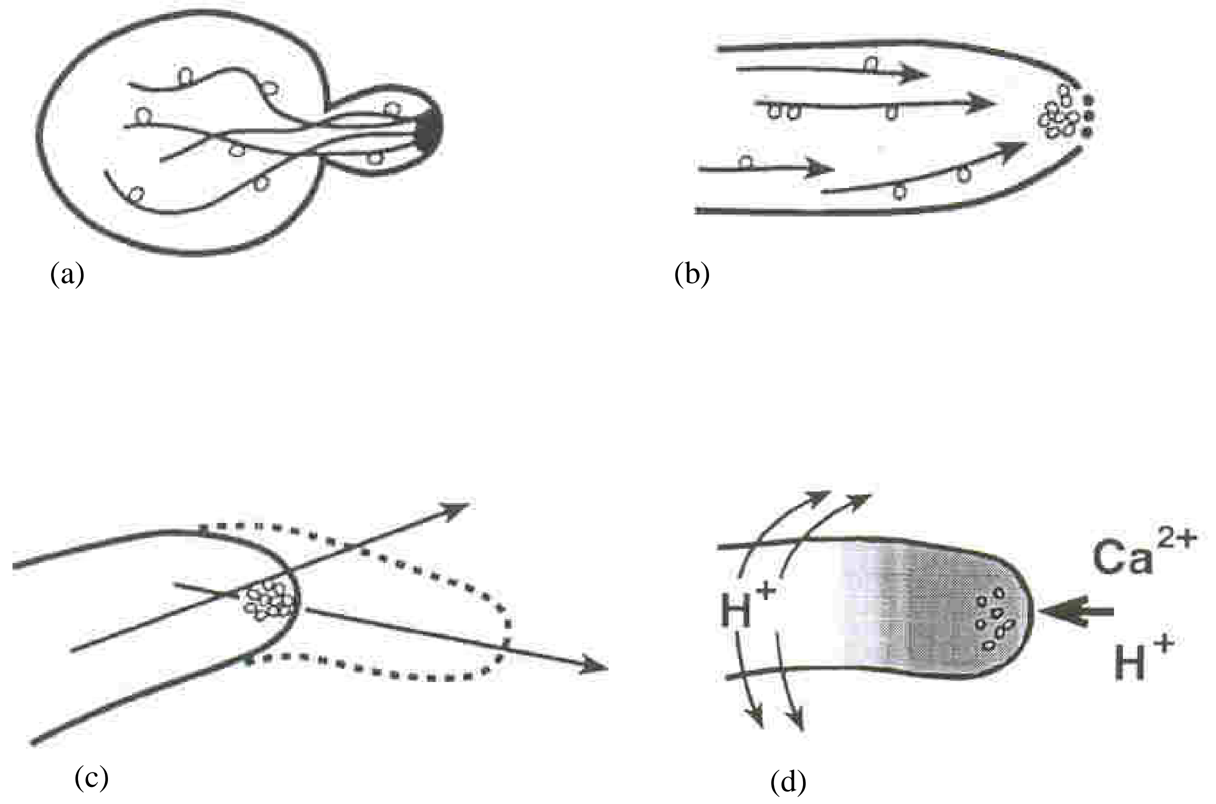


Figure 8

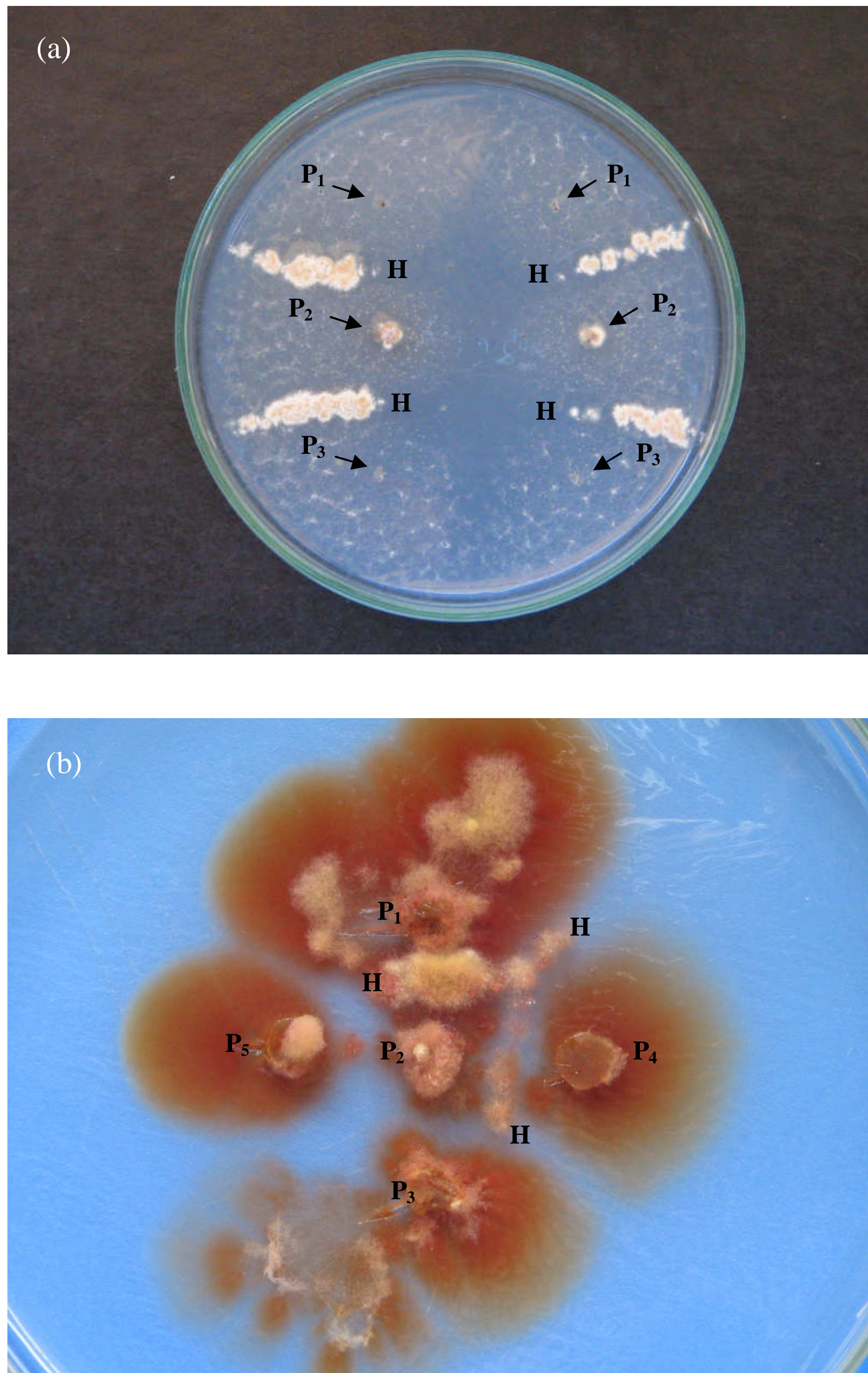


Figure 9

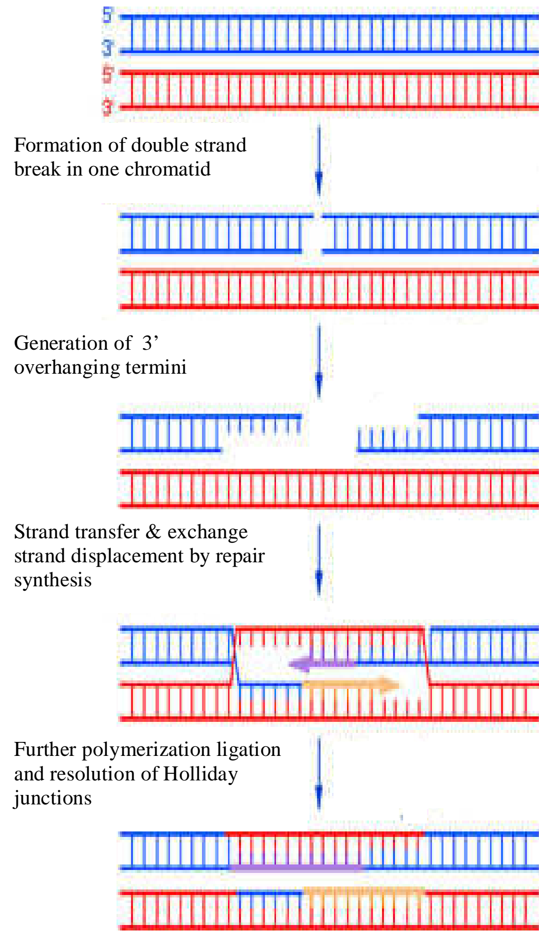
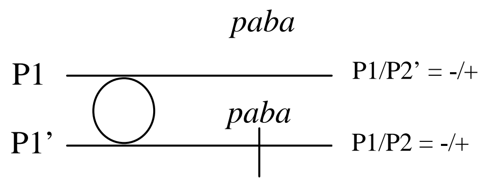
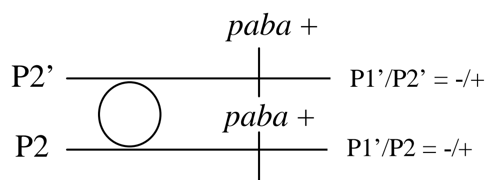


Figure 10

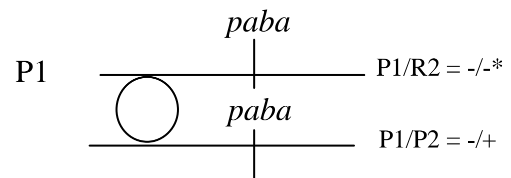
No crossing-over

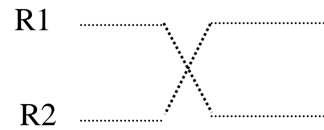


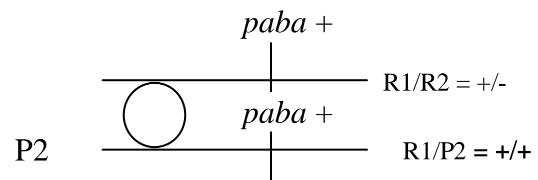


4+ : 4-

One crossing-over event







4+ : 2-

Figure 11

Genotoxic evaluation of sodium nitroprusside in *Aspergillus nidulans*.

Genotoxic evaluation of sodium nitroprusside in *Aspergillus nidulans*.

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Running Title: Genotoxicity in *A. nidulans.*

Key words: nitric oxide, parasexual cycle, homozygotization index, conidiation, mitotic recombination.

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ABSTRACT

The exogenous nitric oxide donor, sodium nitroprusside, evaluated the recombinogenic potential of nitric oxide. Drug inhibited mycelial growth and conidiation in A757 *Aspergillus nidulans* master strain. Two heterozygous diploid strains, one wild ($uvsH^{+}//uvsH^{+}$) and the other defective to DNA repair (*uvsH//uvsH*) were used for recombinagenesis tests. Sodium nitroprusside recombinogenic effect was evaluated by the induction of homozygosis of recessive genes, originally present in heterozygous condition. Results show that sodium nitroprusside (40 μM, 80 μM and 160 μM) is effective in inducing mitotic crossing-over in diploid cells of *A. nidulans.*

INTRODUCTION

The loss of a functional copy of a heterozygous tumor suppressor gene represents an important step during neoplastic transformation. Considered as a somatic event, loss of heterozygosity (LOH) of the long arm of chromosome 13 is the most common mechanism by which the wild-type allele at the retinoblastoma tumor-suppressor locus (*RB1*) is lost in a heterozygous retinal cell for a null mutation. Retinoblastoma may arise from the resulting daughter cell which will be either homozygous or hemizygous for the mutant allele (Hagstrom and Dryja, 1999, Ramel et al., 1996).

Several pathways, including chromosomal deletion, mitotic non-disjunction and mitotic recombination, may lead to LOH (Petek et al., 2003, Lasko et al., 1991). Mitotic recombination consists of exchange events between homologous chromosomes that, following chromosome segregation and cell division, may result in homozygosis of distal genes to the point of exchange (Biggins and Murray, 1999, Beumer et al., 1998). This process plays an essential role in the DNA repair mechanism of eukaryotic cells (Seoighe and Wolfe, 1998, Wolfe and Shields, 1997) and may be initiated by double- or single-strand breaks (Biggins and Murray, 1999, Galli and Schiestl, 1998, Geigl and Eckardt-Sxhupp, 1991).

Although the genetic control of mitotic recombination is poorly understood, it is known to increase in eukaryotic cells in response to a variety of DNA-damaging agents such as ionizing and non-ionizing radiation and chemical substances (Becker et al., 2003, Geigl and Eckardt-Schupp 1991, Takabayashi et al., l984). Intercalating agents such as cryptolepine that induce breaks in DNA and inhibitors of DNA synthesis such as danofloxacin are in fact known as recombinagenic effectors (Chiuchetta and Castro-Prado, 2002, Leonardo and Castro-Prado, 2001).

The free radical gas nitric oxide (NO) acts as a transcellular messenger molecule in both physiological and pathological processes in humans, including inflammation and cancer (Lala and Orucevic, 1998, Moncada et al. 1991, Collier and Vallance, 1989). The gaseous molecule is synthesized by nitric oxide synthase (NOS) from L-arginine (L-Arg) and has been shown to have tumor promoting and inhibitory effects (Xu et al. 2002, Brennan and Moncada, 2002). In the intracellular environment nitric oxide is a highly reactive molecule causing DNA damage via the generation of peroxynitrite (ONOO-), which can cause DNA single-strand breaks, and nitrogen trioxide (N_2O_3), which can cause DNA-crosslinking. One of the consequences of nitric oxide mediated DNA damage is to trigger accumulation of the p53 which may lead to cell cycle arrest and cellular apoptosis, this being a possible pathway by which nitric oxide may exert anti-tumor properties (Xu et al. 2002, Forrester et al. 1996, Wink et al., 1991). Alternatively, nitric oxide generated by NOS may stimulate tumor growth and metastasis by promoting new blood vessel formation through the up-regulation of vascular endothelial growth factor (VEGF), a glycoprotein that causes increased mitosis in endothelial cells (Lala and Chakraborty 2001, Moochhala and Rajnakova, 1999).

Sodium nitroprusside (SN) is a nitric oxide donor which can cause DNA single-strand breaks resulting in mitotic crossing-over. This chemical was recently suggested as a treatment for cerebral ischemia in patients with severe, medically refractory vasospasm after subarachnoid hemorrhage (Raabe et al. 2002). Vasodilating therapy with sodium nitroprusside has also proven effective in patients with left ventricle failure, but arterial hypotension is its main side effect (Bregagnollo et al. 1999).

The filamentous fungus *Aspergillus nidulans* has often been used in mitotic crossing-over studies because this fungus spends a substantial proportion of its life cycle in the G2 phase (Bergen and Morris, 1983). Our research investigated the recombinogenic potential of the nitric oxide donor sodium nitroprusside in diploid *Aspergillus nidulans*.

MATERIAL AND METHODS

Fungal strains and culture media

The origin and the genotypes of the *A. nidulans* strains used in this study are shown in Table I, the DNA repair proficient diploid strain UT448//A757 and the DNA repair deficient diploid strain B211//A837 being prepared according to the method of Roper (1952). Czapek-Dox medium supplemented with 1% (w/v) glucose was used as the Minimal Medium (MM) while the Complete Medium (CM) was that described by Pontecorvo et al. 1953 and Van de Vate and Jansen (1978). Supplemented medium (SM) consisted of MM plus the nutrients required by each strain. Solid medium contained 1.5% (w/v) agar.

Evaluation of sodium nitroprusside toxicity

Conidia of *A. nidulans* haploid strain A757, derived from colonies grown in CM, were inoculated at the center of six CM plates (the control group) and six plates containing CM supplemented with various concentrations (40, 80, 160 or 320μM) of sodium nitroprusside (CM+SN) (the experimental group). The plates were incubated at 37°C and the diameter of the colonies measured after 24, 48, 72, 96 and 120h incubation. The growth rates of the control and experimental groups were compared using the Student's *t*-test at $p < 0.05$.

Spore Production

Conidia from strain A757, derived from colonies grown in CM, were inoculated at the center of six plates containing CM (the control group) and six containing CM+SN (the experimental group) (40 to 320 μM) which were incubated for 24 h at 37°C. After incubation spore production was determined by washing each plate with 15 ml of 0.01% (w/v) Tween 80 and 20 sterilized glass beads (3 mmϕ) to produce a suspension of conidia which were counted

using a haemacytometer. Results for the control and experimental groups were compared using the Student's *t* test at $p < 0.05$.

Calculating Homozygotization Index

Conidia of the diploid *A. nidulans* strains (UT448//A757 and B211//A837) were individually inoculated onto MM+SN plates containing different concentrations of sodium nitroprusside (40, 80 or 160 μM) and incubated for 5 days at 37°C. This treatment produced 19 visible mitotic morphologically identifiable diploid sectors (D1 to D19) which differed from the original diploid strain. The sectors were homozygous (+/+) or heterozygous (+/- or -/+) diploids but never recessive homozygotes (-/-) because these cannot grow on MM. The new diploid strains (D1 to D19) were purified on MM, individually transferred to CM plates and processed by spontaneous haploidization. After haploidization, the haploid mitotic segregants, obtained from diploids D1-D18, were purified in CM and their mitotic stability evaluated. Only mitotically stable segregants at the final stage were selected for the recombinagenesis test (Chiuchetta and Castro-Prado, 2002).

For the recombinagenesis tests conidia of each haploid segregant were individually transferred to 25 positions on CM plates and incubated for 48 h, after which colonies were transferred to different supplemented media: MM supplemented with all of the nutritional requirements of the master strains (UT448 and UT196 or B211 and A837), being omitted one of them, in each type of medium. Mitotic crossing-over causes homozygotization of heterozygous-conditioned genes. If sodium nitroprusside induces mitotic crossing-over in diploid strains UT448//A757 and B211//A837 only heterozygotes (+/- or -/+) or homozygotes (+/+) diploids will develop in Minimal Medium and the nutritional markers will segregate among the haploids in the proportion of 4+ to 2- but if sodium nitroprusside does not induce crossing-over the proportion will be 4+ to 4- because the initial selection process limits the

growth of -/- diploids. The ratio of prototrophic to auxotrophic segregants is described by the Homozygotization Index (HI), a HI value equal to or higher than 2 indicating that sodium nitroprusside has recombinogenic effects (Pires and Zucchi, 1994). The recombinogenic potential of sodium nitroprusside was assessed by comparing the homozygotization indices of the *p*-aminobenzoic acid (*paba*), biotin (*bi*), methionine (*meth*) and pyridoxine (*pyro*) genes using the Chi-squared (χ^2) test with the Yates correction and $p < 0.05$.

RESULTS AND DISCUSSION

We found that, as compared to controls which received no sodium nitroprusside, 40 to 320 μM of sodium nitroprusside modified the mycelial growth of strain A757 and reduced the conidia production (Table 2, Figure 1) but did not affect the temporal development of cells (data not shown), these results being in agreement with those of Ninnemann and Maier (1996) who demonstrated sodium nitroprusside inhibited conidiation by *Neurospora crassa.*

Conidiation in *A. nidulans* requires a transition from the polarized growth pattern of vegetative hyphae to apolar budding of uninuclear cells. The products of at least two major regulatory genes, *brlA* (bristle) and *stuA (*stunted), are transcription factor proteins that regulate the asexual sporulation in *A. nidulans* (Timberlake and Clutterbuck 1994, Wu and Miller, 1997). The *brlA* gene encodes a C_2H_2 zinc finger protein mediating the transition from polarized to radial growth at the stalk apex of the asexual spore-forming conidiophore to form the conidiophore vesicle (Aguirre 1993, Adams et al. 1988) while regulated *stuA* expression is required for correct cell-pattern formation during asexual reproduction (Wu and Miller, 1997). We suggest that alterations in *A. nidulans* conidiation induced by sodium nitroprusside may be due to nitric oxide impairing the transcriptional activation of structural sporulation-specific genes. It has already been shown that nitric oxide inhibits both the DNA binding activity of yeast zinc finger LAC9 and non-zinc finger CPF-1 transcription factors (Kröncke 2001, Berendji et al. 1999, Kröncke et al. 1994).

We evaluated the recombinogenic potential of sodium nitroprusside towards *A. nidulans* diploid strains UT448//A757 (repair proficient) and A837//B211 (repair deficient) using the homozygotization index of various nutritional markers, homozygotization indices for the *bi*, *paba* and *pyro* genes being significantly higher in diploid strains treated with sodium nitroprusside (strains D2 to D4 and D6 to D18) as compared to untreated control

strains (Tables 3 and 4). Diploid strains D1 and D5, derived from strain UT448//A757 treated with sodium nitroprusside, showed homozygotization indices of greater than 2 for the *paba* and *bi* genes but these results were not statistically significant (Table 3). In contrast, however, all the diploid strains obtained after sodium nitroprusside treatment of strain A837//B211 (strains D10 to D18) showed *bi*, *paba* and *pyro* genes homozygotization indices higher than 2 and significantly different from control values (Table 4). Kafer and Mayor (1986) and Yoon et al. (1995) showed that homozygous post-replication repair deficient *A. nidulans uvsH* mutants such as strains A837 and B211 have a high frequency of spontaneous mitotic recombination in condition, a fact which explains the higher sodium nitroprusside sensitivity of the repair deficient diploid strain A837//B211 than the repair proficient strain UT448//A757.

We obtained *meth* gene homozygotization indices of less than 2 for A837//B211 strains D10 to D18 (Table 4) and this may have been due to the greater number of white (*w*) segregants isolated after haploidization, because in *A. nidulans* strain B211 the *w* gene is closely linked to the *meth* gene and consequently most of the *w* segregants have the *meth* phenotype.

Although auxotrophic diploids (-/-) are not selected for in Minimal Media supplemented with sodium nitroprusside, recessive homozygous diploids may be obtained for coloration markers of conidia such as the *w* gene. In our experiments only prototrophic diploids with chartreuse conidia (cha//*cha*) were isolated from diploid B211//A837 treated with sodium nitroprusside, although prototrophic diploids with green (*y+//y*) and white (*w//w*) conidia were isolated from colonies of diploid UT448//A757 treated with sodium nitroprusside. Phenotypic analysis showed diploid D19 (white) to be a recombinant for the centromere-*meth* interval of chromosome II (Figure 2).

The recombinogenic effect of sodium nitroprusside in diploid *A. nidulans* cells may reflect a direct genotoxic effect of nitric oxide on DNA, stimulating the occurrence of DNA breaks during the G2 period. It is also possible that the same genotoxicity effect operates in mammalian cells and may contribute towards the nitric oxide tumor promoting effect.

The transformation of normal human cells into cancer cells is a multistep process and mitotic recombination is a factor that may be involved in the overall transformation process. Since mitotic crossing-over promotes homozygotization of recessive genes, studies have been developed to identify possible recombinogenic agents. Our results show that at concentrations between 40 μM to 160 μM the nitric oxide donor sodium nitroprusside is efficient at inducing mitotic crossing-over in diploid strains of *A. nidulans*.

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TABLES

Table 1. Genotype and origin of *Aspergillus nidulans* strains

Strains	Genotype†	Origin‡
UT448	*riboA1, pabaA124, biA1* (I); *AcrA1, wA2* (II)	Utrecht Stocks
B211	*yA2; biA1; AcrA1; wA2; methA17; uvsH77, pyroA4, chaA1*	LGM
A837	*pabaA1, uvsH77, pyroA4, choA1, chaA1*	FGSC
A757	*yA2, methA17, pyroA4*	FGSC

†Mutant allele phenotypes. Requirement for: riboflavin = *riboA1*; *p*-aminobenzoic acid = *pabaA124* or *pabaA1*; biotin = *biA1*; methionine = *methA17*; pyridoxine = *pyroA4*; and choline = *choA1*. Conidia color: chartreuse = *cha*; *w* = white; and *y* = yellow. Miscellaneous: Acriflavine resistance = *AcrA1*; sensitivity to UV light = *uvsH77*,.
‡FGCS = Fungal Genetic Stock Center, University of Kansas Medical Center, Kansas U.S.A.; LGM = Laboratory of Genetics of Microrganisms, State University of Maringá, Maringá, Paraná, Brazil.

Table 2. Influence of 40 to 320 μM of sodium nitroprusside on conidiation in *Aspergillus nidulans* strain A757.

	Sodium nitroprusside Concentration (μM)				
	0†	**40**	**80**	**160**	**320**
Conidia per mL x 10^3	720.6	235.6*	149.4*	8.1*	7.0*

†Control
*Significantly different from control (Student's *t* test, $p < 0.05$). The arithmetic mean of four experiments was estimated for each treatment.

Table 3. Total number of segregants (NS) and homozygotization Indexes (HI) for markers belonging to *Aspergillus nidulans* DNA repair proficient diploid strain UT448//A757 after treatment with 40 μM (D1-D3), 80μM (D4-D6) or 160μM (D7-D9) of sodium nitroprusside.

Strain[†]	Control[‡]		D1		D2		D3		D4		D5		D6		D7		D8		D9	
	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI
bi+	169	1.41	44	2.00	42	2.63	46	2.88*	45	3.75*	35	2.33	49	3.77*	51	3.64*	40	3.08*	32	1.03
bi	120		22		16		16		12		15		13		14		13		31	
paba+	173	1.49	46	2.30	44	3.14*	43	2.26	41	2.56	36	2.57	48	3.43*	53	4.42*	42	3.82*	51	4.25*
paba	116		20		14		19		16		14		14		12		11		12	
pyro+	150	1.08	35	1.59	32	1.07	29	1.38	35	1.59	29	1.38	32	1.07	42	1.27	30	1.30	35	1.25
pyro	139		22		30		21		22		21		30		33		23		28	

[†]*bi* = biotin; *paba*= *p*-aminobenzoic acid; and *pyro* = pyridoxine.
[‡]Not treated with sodium nitroprusside
*significantly different from control (p< 0.05)

Table 4. Total number of segregants (NS) and homozygotization Indexes (HI) for markers belonging to *Aspergillus nidulans* DNA repair deficient diploid strain A837//B211 after treatment with 40 μM (D10-D12), 80μM (D13-D15) or 160μM (D16-D18) of sodium nitroprusside.

Strain[†]	Control[‡]		D10		D11		D12		D13		D14		D15		D16		D17		D18	
	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI
paba+	73	1.97	55	4.58*	37	2.46	54	4.50*	31	1.72	45	5.00*	40	5.71*	41	5.13*	34	3.78	39	5.57*
paba	37		12		15		12		18		09		07		08		09		07	
bi+	70	1.75	50	2.94	42	4.20*	36	1.20	40	4.44*	44	4.40*	33	2.35	29	1.45	36	5.14*	38	4.75*
bi	40		17		10		30		09		10		14		20		07		08	
meth+	11	0.11	11	0.19	05	0.11	07	0.12	10	0.26	09	0.16	09	0.24	06	0.14	05	0.13	08	0.21
meth	99		56		47		59		39		55		38		43		38		38	

[†]*bio* = biotin; *meth*= methionine; and *paba*= *p*-aminobenzoic acid.
[‡]Not treated with sodium nitroprusside
*significantly different from control (p< 0.05)

FIGURE LEGENDS

Figure 1. Growth of *A. nidulans* strain A757 on plates containing Complete Medium plus various concentrations (0 (control), 40, 80, 160 and 320 μM) of sodium nitroprusside. An asterisk (*) indicates that growth was significantly different from the control (*t*-test, $p < 0.05$).

Figure 2. (A) Mitotic instability of diploid strain D19 obtained after sodium nitroprusside treatment (160 μM) of diploid strain UT448//A757; (B) stable mitotic segregant derived from strain D19.

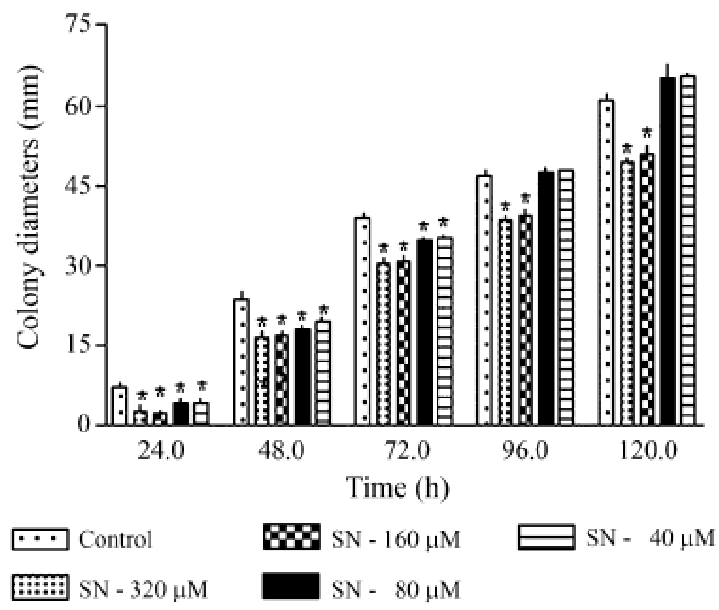

Figure 1

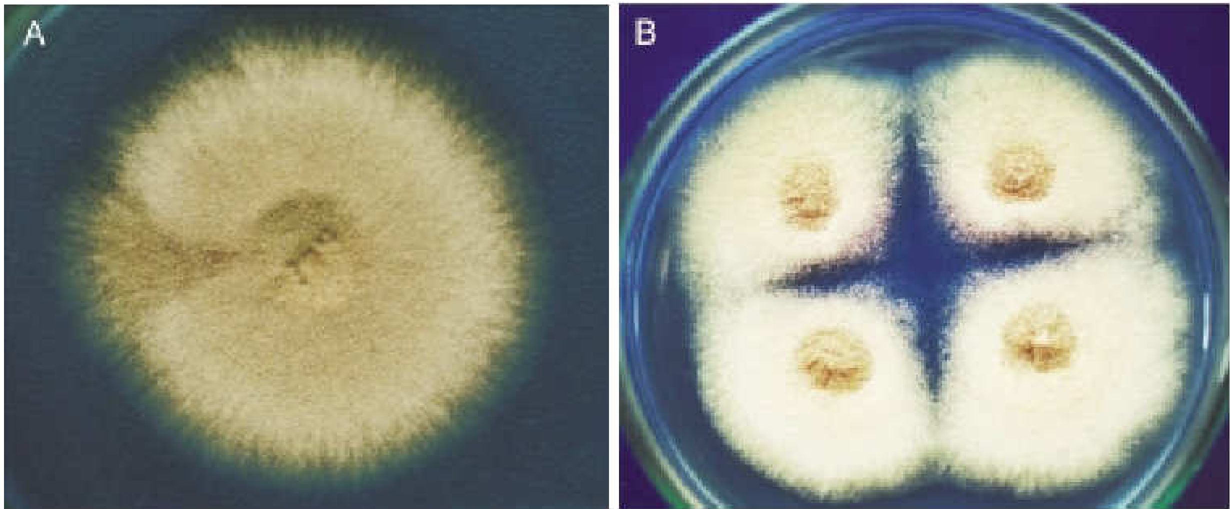

Figure 2

APÊNDICE

Mini Revisão

Parassexualidade em fungos filamentosos: mecanismo, ocorrência e aplicações

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Título Resumido: Ciclo parassexal em fungos

Palavras-chave: parassexualidade, incompatibilidade vegetativa, anastomose, recombinação mitótica, fungo.

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I. RESUMO

Nessa revisão será abordado o processo de parassexualidade em fungos, destacando-se a participação do ciclo parassexual na geração de variabilidade genética. O ciclo parassexual inicia-se com a formação de um heterocário entre linhagens de constituição genética distinta, porém pertencentes ao mesmo grupo de compatibilidade vegetativa. Núcleos diplóides heterozigotos podem originar-se do micélio heterocariótico, os quais poderão seguir três caminhos distintos: a) propagar-se através de mitoses sucessivas, originando novos núcleos diplóides, recombinantes ou não, b) originar núcleos aneuplóides instáveis, até retornar ao estado haplóide normal, através de perdas cromossômicas aleatórias, e c) sofrer recombinação e haploidização ainda no interior da hifa heterocariótica e originar haplóides recombinantes. A formação de um heterocário compatível está sujeita a um rígido controle genético, onde linhagens vegetativamente compatíveis são definidas como membros de um mesmo grupo de compatibilidade vegetativa (VCG). A formação de recombinantes pelo processo de crossing-over mitótico pode ser induzida por agentes físicos e químicos, dentre os quais destacam-se as radiações ionizantes e não-ionizantes, os inibidores da síntese de DNA, e os agentes que promovem alterações no ciclo celular.

II. INTRODUÇÃO

Os Eumycota, ou fungos verdadeiros são microrganismos eucarióticos, quimiorganotróficos, com um só núcleo, como as leveduras, ou multinucleados, como se observa entre os fungos filamentosos. A maioria dos Eumycota apresenta um corpo filamentoso denominado micélio, formado por um conjunto de filamentos anastomosados, em menor ou maior grau, denominados hifas. A anastomose de hifas consiste num estágio importante para a translocação de água e nutrientes entre as hifas e para manter a homeostasia geral da colônia. O crescimento das hifas está associado a um rígido controle genético e a sinais químicos difusíveis, que regulam o processo Spinzenkörper (Gregory 1984, Davidson et al. 1996, Glass et al. 2000).

A anastomose pode ocorrer entre hifas de uma mesma colônia ou entre hifas de fungos geneticamente diferentes, formando um heterocário. Este último é importante para o ciclo de vida de muitos fungos e consiste na primeira etapa do ciclo parassexual (Xiang et al. 2002).

A viabilidade do heterocário depende da constituição genética das linhagens em relação aos loci denominados *het* (incompatibilidade do heterocário) ou *vic* (incompatibilidade vegetativa). Linhagens geneticamente idênticas para um conjunto de loci *het* são capazes de formar heterocários estáveis e são descritas como membros de um mesmo grupo de compatibilidade vegetativa (VCG). Linhagens que diferem em apenas um alelo *het* ou *vic* específico, por outro lado, são incapazes de formar heterocários vegetativamente estáveis e são classificadas como incompatíveis vegetativamente (Saupe 2000).

O heterocário consiste na associação de núcleos haplóides distintos em um mesmo citoplasma. Alguns desses núcleos podem fundir-se, ocasionalmente, formando núcleos diplóides, os quais estão sujeitos a sucessivas divisões mitóticas. Durante essas divisões, dois processos podem ocorrer: não disjunção cromossômica e o crossing-over mitótico (Kafer 1961).

Em muitas espécies de fungos, o núcleo diplóide é dificilmente observado, provavelmente devido a haploidização do núcleo 2n ainda na hifa heterocariótica. Nesse caso, se a recombinação mitótica ocorrer antes da formação dos conídios, recombinantes haplóides poderão ser isolados diretamente do heterocário. Esse processo foi denominado ciclo parassexual com parameiose. (Bonatelli et al. 1983, Becker e Castro-Prado 2004, Becker e Castro-Prado 2005).

A recombinação mitótica associada ao ciclo parassexual pode ser induzida por agentes físicos e químicos que promovem lesões na molécula de DNA, os quais foram descritos como indutores de permutas mitóticas em células eucarióticas. Dentre estes podemos citar as radiações ionizantes e não-ionizantes, os venenos de topoisomerase II tais como doxorrubicina e etoposida, os inibidores da síntese de DNA, tais como danofloxacina, norfloxacina e 5-azacitidina, e os agentes que promovem alterações no ciclo celular tal como a vincristina (Abbadessa e Burdick 1963, Wood e Kafer 1969, Crebelli e Carere 1987, Franzoni et al. 1997, Franzoni e Castro-Prado 2000, Leonardo e Castro-Prado 2001, Chiuchetta e Castro-Prado 2002a,b).

Nessa revisão será abordado o mecanismo da parassexualidade em fungos, e sua importância como ferramenta natural para a geração de variabilidade genética em fungos perfeitos e imperfeitos.

III. CICLO PARASSEXUAL E VARIABILIDADE GENÉTICA

A diversidade genética em fungos está principalmente associada aos processos de mutação e recombinação gênica. Enquanto a mutação consiste numa fonte única de material genético novo às espécies, o ciclo sexual, através da meiose, permite a recombinação das características hereditárias, originando linhagens com genótipos distintos para diferentes marcadores. Em fungos que se reproduzem apenas assexuadamente, por outro lado, linhagens recombinantes podem originar-se mesmo na ausência da meiose, através de um mecanismo de reprodução denominado ciclo parassexual. Fungos que se reproduzem sexuadamente, tais como *Emericella nidulans* (anamorfo: *Aspergillus nidulans*) e *Magnaporthe grisea* (anamorfo: *Pyricularia grisea*) reproduzem-se também através do ciclo parassexual (Baron 1996, Adams et al. 1998, Souza et al. 2003).

A variabilidade genética decorrente da recombinação parassexual foi descrita pela primeira vez por Pontecorvo et al. (1953) em trabalhos realizados com o ascomiceto *A. nidulans.* Posteriormente, o termo parassexualidade foi proposto por Pontecorvo (1956) para definir processos que resultam na obtenção de linhagens recombinantes sem a passagem pelo ciclo sexual.

Caten (1981) listou cerca de quarenta espécies de fungos onde o ciclo parassexual foi detectado. As indicações são de que os processos vegetativos de formação de diplóides e de recombinação somática são quase universais entre os fungos filamentosos, embora existam exceções, como mostram os trabalhos com *Neurospora crassa* (Roper 1966, Azevedo 1998) (Quadro I).

Quadro I: Espécies de fungo cujo ciclo parassexual já foi descrito (extraído de Azevedo 1998)

Ascochya imperfecta	*Metarhizium anisopliae*
Aspergillus amstelodami	*Microsporum gypsium*
Aspergillus flavus	*Penicillium chrysogenum*
Aspergillus fumigatus	*Pencillium digitatum*
Aspergillus nidulans	*Penicillium expansum*
Aspergillus niger	*Penicillium italicum*
Aspergillus oryzae	*Phycomyces blakesleanus*
Aspergillus rugulosus	*Phymatotrichum omnivorum*
Aspergillus sojae	*Phytophthora infestans*
Beauveria bassiana	*Piricularia oryzae*
Cephalosporium acremonium	*Puccinia graminis tritici*
Cephalosporium mycophylum	*Saccharomyces cerevisae*
Cochiobolus sativus	*Schizaphyllum commune*
Coprinus fimetarius	*Trichoderma pseudokoningii*
Coprinus logopus	*Ustilago hordei*
Dyctyostelium discoidem (Mixomiceto)	*Ustilago maydis*
Fusarium oxysporum f. sp. Callistephi	*Ustilago violácea*
Fusaruim oxysporum f. sp. Cubene	*Verticillium albo-atrum*
Fusarium oxysporum f. sp. Pisi	*Verticillium dahlias var. longisporum*

O ciclo parassexual inicia-se com a formação do heterocário, que consiste na existência de núcleos de composição genética distinta em um citoplasma comum. Um micélio heterocariótico é formado através da anastomose de hifas homocarióticas. O processo pode

resultar na fusão de dois núcleos haplóides distintos, no interior das hifas heterocarióticas, originando um núcleo diplóide heterozigoto (Viaud et al. 1998). Em *A. nidulans*, conídios diplóides apresentam freqüência de $1/10^6$ a $1/10^7$ conidios haplóides nos haterocários (Azevedo 1998).

Núcleos diplóides heterozigotos podem originar espontaneamente núcleos diplóides recombinantes, através do crossing-over mitótico, e/ou haplóides recombinantes, através dos processos de recombinação e haploidização (Stern 1936, Roper e Pritchard 1955, Pontocorvo e Kafer 1958, Kafer 1961).

A haploidização consiste na perda sucessiva e aleatória de um membro de cada par cromossômico, através de divisões mitóticas sucessivas. Núcleos aneuplóides, cujo número de cromossomos é diferente do número 2n original (2n +1 ou 2n – 1), serão formados durante a haploidização, através do processo de não-disjunção-cromossômica. Os núcleos aneuplóides, através de novas não-disjunções mitóticas, poderão retornar ao estado haplóide original, contendo novas combinações gênicas, devido à segregação aleatória dos cromossomos ou à ocorrência do crossing-over mitótico antes das perdas cromossômicas (Pacolla-Meirelles e Azevedo 1991, Griffiths et al. 2000, King e Insall 2003, Schoustra et al. 2004) (Figuras 1 e 2).

Em *A. nidulans,* linhagens aneuplóides podem ser macroscopicamente identificadas pela formação de colônias com bordos irregulares e de crescimento mais lento que as colônias haplóides normais. Tais linhagens são também instáveis mitoticamente, originando espontaneamente variantes (ou segregantes) mitóticos em suas colônias (Pollard et al. 1968) (Figura 3).

Durante a mitose as cromátides homólogas podem estabelecer contatos entre si, permitindo trocas recíprocas entre seus segmentos, desencadeando o processo de permuta mitótica. Em eucariotos, este processo é observado em células diplóides heterozigotas, ocorre

durante a interfase e permite a expressão de genes recessivos previamente mascarados pela presença do alelo dominante (Zimmermann 1971, Zimmermann 1992).

Em núcleos diplóides heterozigotos, a segregação das cromátides irmãs durante a mitose é essencial para a detecção do crossing-over mitótico e existem três possibilidades de segregação. Na segregação do tipo X, as cromátides recombinantes segregam para pólos mitóticos opostos, promovendo a homozigose dos genes localizados em posição distal ao ponto de permuta. Esta segregação permite a identificação dos produtos recombinantes pela origem de células filhas fenotipicamente distintas do diplóide original. Na segregação do tipo Z, as cromátides recombinantes segregam para o mesmo polo mitótico, e desta forma, originam células filhas fenotipicamente idênticas às células diplóides heterozigotas originais. A segregação do tipo Y ocorre devido a uma falha na segregação das cromátides irmãs, as quais segregam juntas para a mesma célula filha. Este processo é equivalente à divisão reducional da meiose I e não é observado com freqüência (Figura 4) (Chua e Jinks-Robertson 1991, Beumer et al. 1998).

Em leveduras, os eventos de recombinação mitótica parecem iniciar-se na fase G1 do ciclo celular, antes da replicação do DNA (Wildenberg 1970) e a resolução do crossing over mitótico pode ocorrer antes da duplicação do DNA, através de cortes nas fitas que não participaram do evento de crossing over mitótico. Esse processo origina células filhas fenotipicamente idênticas à célula original. A origem de células homozigotas para os marcadores distais ao ponto de permuta mitótica, somente será possível se os eventos de crossing-over iniciados em G1 forem resolvidos pela replicação do DNA, originando assim os mesmos pares de células com fenótipos recombinantes ou paternais, que resultam do crossing over mitótico iniciado na fase G2 do ciclo celular (Figura 5). O crossing over mitótico em leveduras também pode ocorrer em G2, uma vez que o alinhamento dos cromossomos

associado à recombinação mitótica em G1 é mantido após a replicação do DNA (Esposito 1978, Chua e Jinks-Robertson 1991).

A variabilidade genética decorrente do ciclo parassexual é bastante estudada em várias espécies de fungos, incluindo os ascomicetos, basideomicetos e deuteromicetos, e dentre esses, destacam-se os fungos de importância industrial, de interesse médico e os fitopatogênicos (Viaud et al. 1998, Castrillo et al. 2004). A ocorrência de recombinação genética sem reprodução sexual confere grande importância a este ciclo, tanto em análises genéticas como na obtenção de linhagens diplóides e haplóides recombinantes.

Em análises genéticas, o ciclo parassexual pode ser utilizado no mapeamento gênico e na detecção de translocações cromossômicas espontâneas e induzidas. Em *A. nidulans*, estudos preliminares baseados na caracterização fenotípica de segregantes parassexuais, permitiram a identificação de translocações cromossômicas. Na ausência de translocações, os segregantes mitóticos apresentam ligação entre os marcadores localizados num mesmo cromossomo e segregação independente entre marcadores de cromossomos diferentes. A detecção de ligação completa entre marcadores conhecidos e mapeados em cromossomos distintos, indica a ocorrência de translocação. (Kafer 1962, 1965). Análises semelhantes permitem o mapeamento de novas mutações (Mc Cully e Forbes 1965, Costa et al 2001, Marins e Castro-Prado 2005, Becker e Castro-Prado 2005).

IV. ANASTOMOSE DE HIFAS

Em fungos filamentosos a fusão entre hifas ocorre continuadamente num processo denominado anastomose, formando uma rede de hifas interconectadas denominada micélio (Gregory 1984).

A fusão de hifas é um processo que se observa em várias etapas do ciclo de vida dos fungos filamentosos. Durante a fase vegetativa, a fusão pode ocorrer inicialmente entre esporos em germinação, e posteriormente no interior da colônia vegetativa madura. Durante a fase sexual, hifas de *mating-type* opostos, no caso de fungos heterotálicos, devem sofrer anastomose. A manutenção da fase dicariótica, que é um pré-requisito para a cariogamia, também requer a fusão de hifas e envolve a formação estruturas especializadas, tais como os *croziers* (ou ganchos) nas hifas ascógenas dos ascomicetos. No ciclo sexual, o processo de anastomose também está envolvido na formação de agregados de hifas dos quais derivam os corpos de frutificação (Glass et al 2004).

Durante a anastomose, as hifas estabelecem contatos umas com as outras através da quebra da parede celular seguida pela fusão das membranas plasmáticas. A fusão pode ocorrer não somente entre hifas de uma mesma colônia, mas também entre hifas de colônias diferentes, resultando na formação do heterocário que, por sua vez, está sujeito ao controle dos sistemas de compatibilidade vegetativa (Xiang et al. 2002, Glass e Kaneko 2003).

IV.I. CONTROLE FISIOLÓGICO DA ANASTOMOSE DE HIFAS

Embora a anastomose de hifas seja um evento contínuo, três estágios fisiológicos distintos podem ser observados durante a fusão: pré-contato, pós-contato e pós-fusão (Figura 6) (Glass et al. 2000).

O estágio de pré-contato envolve o crescimento e o direcionamento da extremidade de uma hifa que irá estabelecer contato com outra hifa da mesma colônia ou de uma colônia diferente. A extremidade de uma hifa em crescimento contém um complexo denominado Spizenkörper, ou corpo apical, formado por um conjunto de vesículas ligadas à membrana e envolvidas por uma rede de microfilamentos de actina. O corpo apical está presente somente nas extremidades das hifas em crescimento, desaparecendo quando este crescimento é

interrompido e reaparecendo novamente quando o crescimento é retomado (Bartinick-Garcia et al 1995a, Glass et al. 2000, Glass et al. 2004).

As vesículas que formam o corpo apical (Figura 7) são provavelmente formadas no Golgi e então, transportadas para o ápice da hifa em crescimento, pelos elementos do citoesqueleto, tais como microtúbulos e microfilamentos de actina e miosina. As vesículas, posteriormente, fundem-se à membrana plasmática da hifa em crescimento, liberando seus conteúdos, os quais certamente variam entre os diferentes tipos de vesículas, e provavelmente incluem: enzimas envolvidas na síntese de parede celular, tais como quitina sintase e glucano sintetase, enzimas envolvidas na lise de parede, enzimas ativadoras e alguns polímeros pré-formados de parede celular, tais como as manoproteínas. Acredita-se, porém, que a maioria dos polímeros de parede seja sintetizada *in situ*, ou seja, na própria extremidade da hifa (Howard 1981, Bartnicki-Garcia et al. 1995a, b).

O influxo de íons Ca^{2+} na célula gera uma alta concentração desse íon nas extremidades das hifas, determinando assim a localização dos pontos de exocitose. Tal influxo é um processo contínuo e permite que as membranas celulares das vesículas secretoras sejam fusionadas à membrana plasmática da célula a fim de descarregarem seus conteúdos (Figura 8) (Jackson e Heath 1993, Regalado 1998).

A parede celular da região em crescimento é, provavelmente, fina e estruturalmente fraca, permitindo que novos componentes de parede sejam inseridos a ela. Acredita-se que a integridade estrutural da extremidade da hifa em crescimento seja dependente da rede de microfilamentos de actina (Deacon 1997).

O crescimento da hifa é um processo extremamente complexo, com a participação coordenada de componentes distintos, de tal forma que alterações no balanço destes componentes poderá alterar a forma ou a direção do crescimento da hifa (Deacon 1997).

No evento denominado pós-contato o processo Spitzenkörper ainda persiste. Inicialmente ocorre adesão entre as paredes celulares das hifas, acompanhada pela parada do crescimento das extremidades. Posteriormente, ocorre a destruição da parede celular, provavelmente devido à liberação de enzimas hidrolíticas no ponto de contato entre as hifas, e ocorre então a fusão das membranas plasmáticas. A formação de uma nova parede celular entre as hifas envolvidas na anastomose está relacionada com a liberação do conteúdo das vesículas que fazem parte do complexo Spitzenkörper (Figura 6) (Hickey et al. 2002).

No evento denominado pós-fusão, o processo de Spitzenkörper desaparece e ocorre a mistura dos conteúdos nucleares e citoplasmático das hifas (Lópes-Franco e Bracker 1996).

IV.II. CONTROLE GENÉTICO NA ANASTOMOSE DE HIFAS

A fusão das hifas vegetativas também pode ser regulada geneticamente. Várias hipóteses sugerem que a maquinaria envolvida na fusão celular em *Saccharomyces cerevisae* pode também estar presente em outros ascomicetos. De fato, muitos dos genes envolvidos na fusão celular em *S. serevisae* são bem conservados em *N. crassa* (Galagan 2003).

Os genes *PMR1, PEA2* e *AGA1,* importantes na regulação da fusão celular em *S. cerevisae,* e conservados em *N. crassa,* codificam, respectivamente, proteínas que atuam na fusão da membrana plasmática, na polarização do citoesqueleto para o deslocamento das vesículas no processo Spitzenkörper, e na síntese e remodelamento da parede celular (Heiman e Walter 2000, Valtz e Herskowitz 1996).

Alguns genes requeridos para a fusão celular em *S. cerevisae,* por outro lado, parecem não ser bem conservados no genoma de *N. crassa,* incluindo *FUS1* e *FUS2.* As funções específicas das proteínas produzidas por esses genes ainda não estão bem definidas, porém a localização destas na zona de fusão celular sugere a participação destas proteínas no referido processo (Gammie et al. 1998, Glass et al. 2004).

V. HETEROCARIOSE E GRUPOS DE COMPATIBILIDADE VEGETATIVA

A formação do heterocário através da anastomose de hifas homocarióticas é um processo importante no ciclo de vida de várias espécies de fungos, atuando como um pré-requisito para a ocorrência dos processos de reprodução sexual e parassexual (Jacobson et al. 1998, Xiang e Glass 2004). A heterocariose permite a troca de material genético e citoplasmático entre linhagens geneticamente distintas, bem como a transferência de fatores de hipovirulência como o (ds) RNAs (RNA de fita dupla) (Van 1982, Nuss e Koltin 1990). O processo de formação do heterocário, em muitos fungos, parece ser limitado pela barreira de incompatibilidade vegetativa (Corner e Poulter 1989, Saupe 2000).

Em algumas espécies de fungos, heterocários sexuais e vegetativos podem ser completamente distintos. Linhagens capazes de formar um heterocário sexual podem ser incapazes de formar um heterocário vegetativo e vice-versa, sendo classificadas como sexualmente compatíveis ou vegetativamente compatíveis, respectivamente. A compatibilidade do heterocário é governada por um rígido sistema genético onde, linhagens vegetativamente compatíveis são incluídas em um mesmo grupo de compatibilidade vegetativa (VCG) (Figura 9). Linhagens sexualmente compatíveis, por outro lado, são governadas por um ou mais loci denominados *mating-type* que podem apresentar um ou mais alelos (Fincham et al. 1979, Glass e Kuldau 1992).

Dois tipos de sistemas genéticos foram descritos como reguladores da compatibilidade vegetativa, o sistema alélico e o não alélico. No primeiro, linhagens idênticas para um conjunto de loci denominados *het* (incompatibilidade do heterocário) ou *vic* (incompatibilidade vegetativa) (Perkins 1988, Saupe 2000) são capazes de formar um heterocário vegetativo estável e compartilham um mesmo VCG. Alternativamente, linhagens que diferem entre si em qualquer um destes loci (*het* ou *vic*) são incapazes de formar heterocário e são classificadas como vegetativamente incompatíveis. No sistema não-alélico,

o alelo de um determinado locus interage com o alelo de um outro locus, bloqueando a formação do heterocário (Chase e Ullrich 1990, Saupe 2000).

O sistema de compatibilidade vegetativa atua, portanto, no sentido de restringir a transferência nuclear e de elementos citoplasmáticos durante o crescimento do fungo. Geralmente a fusão de hifas (anastomose) ocorre normalmente entre linhagens portadoras de núcleos vegetativamente incompatíveis, entretanto, após a fusão incompatível, ocorre a morte do heterocário, por um mecanismo não compreendido até o presente momento (Leslie 1993).

Em *N. crassa* dez diferentes loci *vic* já foram identificados e mapeados em cinco dos sete cromossomos existentes nesta espécie. Dois desses loci apresentam alelos múltiplos (Leslie 1987). Oito loci *het* são conhecidos no fungo *A. nidulans,* sendo que alelos múltiplos estão presentes em pelo menos dois destes loci (Croft e Dales 1984). Em *Fusarium moniliforme*, com base na segregação de diferentes VCGs, em cruzamentos de linhagens específicas, dez loci *vic* foram identificados (Puhalla e Spieth 1985).

O fungo *Cryphonectra pasasitica* é o agente etiológico da doença do castanheiro, que promove a formação de cancro ou tumores letais na casca das árvores dos castanheiros, além de destruir o sistema vascular da planta. Linhagens hipovirulentas de *C. parasítica* são infectadas com vírus de (ds) RNAs, os quais encontram-se localizados no citoplasma das células. O (ds) RNA viral pode ser transmitido de uma linhagem para outra através da heterocariose, o que constitui um processo natural de controle biólógico da doença do castanheiro. A transmissão do fenótipo hipovirulento, entretanto, pode ser limitada pelo sistema de incompatibilidade vegetativa do fungo *C. parasítica* que envolve pelo menos sete loci *vic.* Linhagens pertencentes a diferentes VCGs já foram de fato isoladas (Anagnostakis e Waggoner 1981, Anagnostakis 1982, Kuhlman e Bhattacharyya 1984, Bissegger et al. 1996).

Três métodos podem ser utilizados na identificação dos loci *vic*: a) avaliação direta da formação do heterocário (geralmente pela complementação entre marcadores genéticos); b)

avaliação direta da inabilidade de formar um heterocário (formação de barreiras); e c) caracterização de linhagens que apresentam regiões duplicadas no genoma e são heterozigotas para um ou mais loci *vic* (Leslie 1993).

Os testes de avaliação direta da heterocariose envolvem geralmente a formação de um heterocário prototrófico estável nas condições em que nenhuma das duas linhagens auxotróficas pode sobreviver. Em princípio, qualquer marcador genético pode ser usado para esta avaliação, porém, na prática, marcadores nutricionais, de resistência a fungicidas ou de coloração de conídios são preferencialmente utilizados pela facilidade de caracterização fenotípica do heterocário. Em qualquer caso, deve-se considerar que heterocários prototróficos serão obtidos somente se as linhagens envolvidas forem idênticas quanto aos loci *het* ou *vic*, caso contrário, o crescimento prototrófico não ocorrerá (Figura 9) (Puhalla e Spieth 1985, Leslie 1993).

A formação de barreiras quanto ao desenvolvimento do heterocário ocorre entre linhagens vegetativamente incompatíveis, através da interação antagonista de suas hifas. A "barreira" forma-se na região de contato entre as duas colônias e pode ser visualisada pela formação de uma região de pigmentação escura, contendo células mortas (Newhouse e MacDonald 1991).

A identificação de loci *vic* pode ainda ser realizada através da obtenção de linhagens portadoras de duplicações das regiões cromossômicas que abrigam genes de incompatibilidade vegetativa. Isto é possível através da construção de linhagens heterozigotas para um locus particular. Tais linhagens são fenotipicamente aberrantes, apresentando crescimento, pigmentação e morfologia anormais (Perkins 1975, Mylyk 1976).

A caracterização de linhagens através da determinação dos grupos de compatibilidade vegetativa permite a identificação de clones provenientes de um progenitor comum, uma vez que linhagens pertencentes a um mesmo VCG são geneticamente semelhantes. Este método

auxilia também a identificação de linhagens patogênicas e no mapeamento gênico (Chaisrisook e Leslie 1990, Desjardins et al. 1992, Leslie 1993).

A utilização de VCGs como ferramenta de diagnóstico é de extrema validade para os fitopatologistas, uma vez que sua aplicação está relacionada com a hipótese de que linhagens pertencentes ao mesmo grupo patogênico integram um determinado VCG (Puhalla e Spieth 1985).

A caracterização de fungos fitopatogênicos em grupos de compatibilidade vegetativa permite a identificação de sub-grupos distintos em populações de fungos previamente considerados genotipicamente homogêneos através de análises moleculares (Busso e Castro-Prado comunicação pessoal, Freeman et al. 2000).

VCGs foram utilizados também na determinação da origem clonal de linhagens de *Fusarium moniliforme* portadoras das mutações *pal1* e *fum1*. As linhagens foram separadas em VCGs distintos, descartando-se a hipótese de origem clonal (Desjardins et al. 1992).

A barreira da incompatibilidade vegetativa, que impede a formação do heterocário, pode ser superada, artificialmente, pela técnica de fusão de protoplastos, células cuja parede celular foi removida enzimaticamente. A técnica consiste num método valioso para a obtenção de híbridos intra e inter-específicos em várias espécies de fungos (Shubha 2004, Corner e Poulter 1989). A fusão intra-específica de protoplastos é um método eficiente para induzir o ciclo parassexual, permitindo a obtenção de novas linhagens através de recombinação mitótica (Anne 1983, Furlaneto e Pizzirani-Kleinr 1992).

VI. CICLO PARASSEXUAL COM PARAMEIOSE

Em algumas espécies de fungos, tais como *Beauveria bassiana, Trichoderma pseudokoningii* e *Cephalosporium acremonium*, os núcleos diplóides formados no interior das hifas heterocarióticas são altamente instáveis, sofrendo haploidização antes da produção de

conídios. Este processo, denominado ciclo parassexual com parameiose, permite a obtenção de recombinantes diretamente do micélio heterocariótico, sem a recuperação da fase diplóide (Bonatelli et al. 1983, Bagagli et al. 1991, Pacolla-Meirelles e Azevedo 1991, Ball e Jamlyn 1982). Embora a parameiose tenha inicialmente sido descrita em fungos imperfeitos, o processo foi recentemente descrito em fungos com fase sexual tais como *Emericella nidulans* (anamorfo: *Aspergillus nidulans*) e *Glomerella sublineolum* (anamorfo: *Colletotrichum sublineolum)* (Baptista et al. 2003, Souza-Paccola et al. 2003).

Em A. *nidulans*, mutantes sensíveis à radiação UV (*uvs*) foram utilizados para formar heterocários com o objetivo de se isolar segregantes parameióticos. Entre as mutações *uvs* deste fungo encontram-se aquelas que aumentam o índice de recombinação mitótica intragênica (*uvsH* e *uvsF*) e aquelas que alteram a segregação cromossômica (*uvsC* e *uvsE*). Heterocários homozigotos e heterozigotos para estas mutações permitiram o isolamento de dois grupos de segregantes mitóticos: haplóides estáveis (parameióticos) e aneuplóides. Os fenótipos recombinantes dos segregantes haplóides foram caracterizados através da análise de marcadores nutricionais, de resistência a Acriflavina e de coloração de conídios. Heterocários formados com mutantes *uvs* produziram 20,8 % de segregantes parameióticos. O grupo controle, representado por heterocários formados com linhagens *uvs+,* originou 4,55% de segregantes parameióticos. Todos os segregantes classificados como parameióticos apresentaram-se estáveis mitótica e meioticamente (Baptista et al. 2003).

Recombinação genética sem ciclo sexual foi também demonstrada no fungo *C. sublineolum,* agente causador da antracnose em *Sorghum bicolor* (L.) Moench. O cruzamento entre mutantes auxotróficos e de resistência ao Benomil e a Ciclohexamina confirmaram a ocorrência da parameiose nessa espécie (Souza-Paccola et al. 2003).

A parameiose pode também ser utilizada como uma ferramenta na investigação do efeito recombinagênico de drogas que afetam o DNA (Becker e Castro-Prado 2004). A

doxorrubicina é um agente antineoplásico capaz de ligar-se ao DNA e inibir a ação da enzima topoisomerase II. A droga apresenta efeito recombinagênico, sendo portanto capaz de promover malignidades secundárias (Noviello et al. 1994, Chiuchetta e Castro-Prado 2002). Heterocários formados entre linhagens *uvs* e *uvs+* de *A. nidulans* foram formados e inoculados em meios seletivos contendo doxorrubicina. A freqüência de segregantes parameióticos obtida foi significativamente maior em presença de doxorrubicina do que em sua ausência (Becker e Castro-Prado 2004).

A parameiose consiste num mecanismo natural de recombinação e variabilidade genética, e pode ser utilizada como uma ferramenta em estudos genéticos e na obtenção de linhagens de interesse comercial (Bagagli et al. 1991).

VII. INDUÇÃO DO CROSSING-OVER MITÓTICO

O crossing-over mitótico constitui um dos principais mecanismos de reparo do DNA em eucariotos e pode ser induzido por agentes físicos e químicos (Haynes e Kunz 1981, Pires e Zucchi 1994). Mutantes de leveduras defectivos na recombinação mitótica não são hábeis em reparar danos no DNA, especialmente quebras em fitas duplas (DSBs) (Resnick e Martin 1976, Ho 1975).

Para reparar danos no DNA através do processo de recombinação, a célula deve conter uma segunda região no genoma que tenha homologia com a região danificada. Em células diplóides, as moléculas de DNA estão presentes na forma de cromossomos homólogos, já em células haplóides, o homólogo é providenciado pela cromátide irmã, presente na fase G2 do ciclo celular, após a replicação do DNA (Esposito 1978, Fabre 1978, Galli e Schiestl 1998).

No reparo recombinacional as fitas que sofreram os cortes iniciais geram uma extremidade 3` OH livre que invade uma região homóloga criando uma alça-D (região de

DNA heteroduplex). A extremidade 3` OH invasora é utilizada como um primer, sendo estendida pela síntese de DNA. A alça-D, por sua vez, permite o reparo da fita complementar. Dessa forma, formam-se duas junções de Holliday que serão resolvidas por clivagem endonucleotídica, originando cromossomos paternais ou recombinantes (Figura 10) (Davies et al. 1975).

Dentre os agentes físicos que induzem o crossing-over mitótico podemos citar as radiações ionizantes e não-ionizantes. As radiações ionizantes, como os raios-X, promovem quebras em fitas duplas de DNA, já a radiação não-ionizante, como a luz ultra-violeta (UV), promove a formação de dímeros ciclobutanos de pirimidinas (CPD) e 6-4 fotoprodutos (6-4PP) na molécula de DNA (Abbadessa e Burdick 1963, Wood e Kafer 1969).

As quebras geradas pelos raios-X podem ser reparadas tanto pelo reparo por excisão quanto pela via de reparo recombinacional (Haynes e Kuns 1981,Kupiec e Steinlauf 1997). Em *S. cerevisae*, genes do grupo epistático *RAD 52* (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11/RAD58*, XRS2 e *RDH54/TID1*) são requeridos para o reparo de danos induzidos pela radiação ionizante. Mutantes *RAD 52* são deficientes na recombinação mitótica e conseqüentemente deficientes no reparo das lesões DSBs. Homólogos aos genes do grupo epistático *RAD 52* já foram identificados em outros eucariotos, incluindo mamíferos, indicando a alta conservação desses genes na via de reparo recombinacional (Ho 1975, Davis e Symington 2004).

A associação do efeito da luz ultravioleta (UV) com o processo de recombinação mitótica foi descrito inicialmente em leveduras por James (1955) e desde então, vários estudos têm demonstrado o efeito recombinagênico desta radiação em células eucarióticas (Wood e Kafer 1969, Pires e Zucchi 1994). As lesões induzidas pela luz UV (CPD e 6-4PP) resultam em consideráveis distorções na estrutura da dupla hélice, estimulando a ação dos mecanismos de reparo (Michell e Nairm 1989).

Em *A. nidulans*, os mutantes deficientes para o reparo do DNA foram divididos em quatro grupos epistáticos: (1) Uvs F/H (*uvsF, uvsH* e *uvsJ*), (2) UvsB (*uvsB* e *uvsD*), (3) UvsC (*uvsC, uvsE, musK, musL* e *musN*) e (4) UvsI (*uvsI*) O primeiro grupo de mutantes caracteriza-se por apresentar níveis normais de recombinação meiótica, porém elevados níveis de recombinação mitótica. Mutantes do segundo grupo apresentam alterações no controle do ciclo celular e originam segregantes instáveis a partir de conídios diplóides. Mutantes do grupo epistático UvsC são deficientes no reparo recombinagênico e hiper-sensíveis à drogas que induzem DSBs. Finalmente, os mutantes do grupo UvsI apresentam-se deficientes no reparo mutagênico (Kafer e Mayor 1986, Goldman et al. 2002).

Linhagens diplóides de *A. nidulans*, homozigotas para a mutação *uvsH* (grupo UvsF/H) foram utilizadas para avaliar a atividade genotóxica do cremofor EL, solvente orgânico utilizado em protocolos de quimioterapia como solubilizante de drogas hidrofóbicas e como emulsificante na indústria de alimentos. Diplóides *uvsH//uvsH* tratados com cremofor EL mostraram aumento significativo na freqüência de crossing-over mitótico quando comparados com linhagens não tratadas (Busso e Castro-Prado 2004).

Pires e Zuchi (1994) descreveram um método para detectar o potencial genotóxico de substância químicas, utilizando o ciclo parassexual do ascomiceto *A. nidulans*. O método consiste em obter diplóides prototróficos, porém heterozigotos para mutações letais condicionais, utilizando-se, especificamente, marcadores nutricionais. Os diplóides são cultivados em Meio Mínimo suplementado com o agente químico em estudo. O Meio Mínimo é constituído apenas por sais minerais, nitrato de sódio e glicose, de maneira a permitir somente o crescimento de linhagens prototróficas.

No meio de cultura, os núcleos diplóides passarão por mitoses sucessivas e originarão novos núcleos diplóides, os quais, por sua vez, poderão ser heterozigotos (+/- ou -/+) ou

homozigotos (+/+) para um determinado marcador genético, porém núcleos homozigotos para genes recessivos (-/-) serão incapazes de se desenvolver em MM.

A instabilidade somática de núcleos diplóides de *A. nidulans* encontra-se geralmente associada a perdas de material genético durante a haploidização ou à ocorrência de crossing-over mitótico (Gabrielli e Azevedo, 1980; Geogpoulos et al. 1976; Parag e Roper, 1975). Tal instabilidade manifesta-se pela origem de setores (ou segregantes) mitóticos nas colônias de linhagens diplóides. Os segregantes mitóticos poderão ser haplóides, aneuplóides ou diplóides e apresentar auxotrofias (Figuras 2 e 3) (Pontecorvo e Roper 1953; Pontecorvo e Kafer 1958).

Para os testes de genotoxicidade, os segregantes diplóides prototróficos obtidos em presença do composto em estudo são purificados em MM e haploidizados em meio de cultura enriquecido (Meio Completo, Van de Vate e Jansen 1978). Os haplóides então obtidos são analisados fenotipicamente quanto aos marcadores nutricionais. O Índice de Homozigotização (HI) para cada marcador analisado será o quociente entre o número de segregantes prototróficos e o número de segregantes auxotróficos. Se a substância em teste induziu crossing-over no diplóide original, espera-se HI $\geq$ 2.0. Se, por outro lado, a substância não induziu recombinação mitótica, o HI será inferior ao valor 2.0 (Figura 11).

Substâncias que alteram os processos de duplicação e transcrição do DNA, tais como doxorubicina e etoposida, estimulam o crossing-over mitótico em células eucariotas (Chiuchetta e Castro-Prado 2002a). Tais compostos são denominados “venenos” de topoisomerase II (Topo II) pois ligam-se à molécula de DNA e inibem a atividade catalítica da enzima Topo II, cuja função é essencial na manutenção da topologia do DNA (Crebelli e Carere 1987, Boege 1996, Becker et al. 2003)

Substâncias que promovem alterações no ciclo celular, como o antineoplásico vincristina, também podem induzir o processo de recombinação somática (Chiuchetta e Castro-Prado 2002b). Vincristina é um alcalóide que se liga especificamente a tubulina,

danificando o aparato mitótico através da despolimerização dos microtúbulos e promovendo a parada do ciclo celular (Blajeski et al. 2002, Holgersson et al. 2005).

O composto 5-azacitidina (5-AC) é um análogo da citidina que apresenta um átomo de nitrogênio na posição 5 do anel pirimídico. Este agente quando incorporado ao DNA, em substituição à citosina, inibe a ação da enzima metiltransferase, e causa hipometilação da molécula de DNA, o que pode resultar na ativação de genes silenciados pela metilação de bases ou pela condensação da cromatina (Rizwana e Hahn 1999, Haaf 1995).

O efeito recombinagênico da 5-AC foi demonstrado em *A. nidulans* utilizando-se uma linhagem diplóide heterozigota para marcadores nutricionais, de coloração de conídios e de resistência à Acriflavina. Segregantes mitóticos foram isolados do diplóide tratado com 5-AC e seus fenótipos foram analisados. 5-AC mostrou-se efetivo na indução do crossing-over mitótico, aumentanto a freqüência de recombinação entre marcadores dos cromossomos I e II da linhagem diplóide (Franzoni e Castro-Prado 2000).

Alterações genéticas múltiplas, tais como mutações de ponto, translocations cromossômicas e perda de heterozigosidade são processos envolvidos no desenvolvimento de neoplasias. Em células heterozigotas para um gene supressor de tumor, a perda da heterozigozidade pode ser resultante de um evento de permuta mitótica que originará clones de células homozigotas para o alelo não-funcional. (Barrett 1993, Ramel et al. 1996). Em função disto, o estudo da genotoxicidade de substâncias químicas torna-se primordial, no sentido de se detectar aquelas que apresentam potencial recombinagênico. O ciclo parassexual de *A. nidulans* tem se apresentado como ferramenta valiosa na identificação de agentes recombinagênicos.

VIII. PERSPECTIVAS

O ciclo parassexual foi descrito em um grande número de fungos filamentosos incluindo aqueles de importância médica e industrial, tais como *A. fumigatus* e *A. niger,* fungos entomopatogênicos e patógenos de plantas, tais como *M. anisopliae* e *V. dahliae.*

Para a análise genética de fungos sexuais e assexuais, a recombinação parassexual vem sendo extensivamente utilizada. Trocas parassexuais de informação genética entre linhagens permitem a eliminação de mutações deletérias, a reconstituição do genoma e a geração de novos variantes genotípicos.

Diplóides com genótipos específicos podem ser selecionados a partir de heterocários resultantes da fusão aleatória de núcleos vegetativos. Tais diplóides podem auxiliar na identificação de interações entre pares de alelos de um mesmo gene ou entre pares de mutações em loci distintos.

O grau de instabilidade mitótica de linhagens diplóides de fungos perfeitos e imperfeitos, tais como *M. grisea* e *V. albo-atrum*, dá suporte à hipótese de que o ciclo parassexual constitui importante fonte de variabilidade genética. Evidências diretas de trocas parassexuais na natureza podem ser obtidas em estudos moleculares da estrutura genotípica de populações.

Após a haploidização de um núcleo diplóide, os haplóides recombinantes obtidos fornecem informações sobre marcadores localizados em cromossomos diferentes (recombinantes inter-cromossomais, resultantes da segregação independente dos cromossomos), ou sobre a ordem dos genes em um cromossomo em relação ao centrômero (recombinantes intra-cromossômicos, resultantes de crossing-over). Dessa maneira, a recombinação parassexual pode ser utilizada no mapeamento mitótico de mutações, aberrações cromossômicas e translocações. O alinhamento dos genes nos cromossomos

produz um mapa genético que fornece a base para o mapeamento cromossômico através da técnica de eletroforese em campo-pulsado (*pulsed-field gel electrophoresis*).

A utilização efetiva de marcadores moleculares no monitoramento da recombinação parassexual, tanto *in vitro* quanto em populações naturais de fungos, trará novos conhecimentos a respeito do mecanismo e do significado evolutivo da parassexualidade.

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X. LEGENDAS DAS FIGURAS

Figura 1: Representação esquemática da segregação normal dos cromossomos na mitose e da segregação dos cromossomos no evento de não disjunção mitótica (modificado de Griffits et al. 2002).

Figura 2: Linhagem diplóide do fungo *Aspergillus nidulnas* revertendo para núcleos haplóides (seta) através do processo de haploidização.

Figura 3: (a): Colônias aneuplóides do fungo *A. nidulans* macroscopicamente distinguíveis pela produção de setores haplóides (setas) através da perda de um dos cromossomos homologos. (b) e (c): Colônias aneuplóides do fungo *A. nidulans* macroscopicamente distinguíveis pela presença de bordos irregulares. (d): Colônias haplóides do fungo *A. nidulans* apresentando ausência de setores.

Figura 4: Representação esquemática da segregação das cromátides irmãs durante a mitose (modificado de Beumer et al. 1998).

Figura 5: A resolução do crossing over iniciado na fase G1 do ciclo celular antes (a) ou após (b) a replicação do DNA originará células filhas homozigotas (c) ou heterozigotas (d) para os genes distais ao ponto de permuta (modificado de Chua and Jinks-Robertson 1991).

Figure 6. Estágios da anastomose de hifas. (a): Pré-contato. Os pontos claros representam o Spitzenkörper. (b): Pós-fusão, o Spitzenkörper persiste e ocorre adesão das paredes celulares das hifas envolvidas na anastomose. Barra representa 10µm (extraído de Glass et al. 2004).

Figure 7. Modelo simplificado do crescimento da hifa. G= Golgi; V= vesícula, m= microtúbulos. (Extraído de Deacon 1997).

Figura 8. (a): Vesículas secretoras de *S. cerevisae* direcionadas pelos microfilamentos de actina, ao sítio de exocitose. (b): Vesículas secretoras de fungo filamentoso direcionadas à extremidade da hifa pelo Spitzenkörper, onde sofrem exocitose. (c): Trajetória do Spitzenkörper determinando a direção do crescimento da hifa. (d): Gradientes citoplasmáticos de íons Ca^{++} ou prótons, definindo o local de exocitose das vesículas (modificado de Harold, 2002).

Figura 9. Formação de heterocários entre linhagens vegetativamente compatíveis de *A. nidulans* (a) e *F. graminearum* (b) inoculadas em meio seletivo. P_1 a P_5 = linhagens paternais, H = heterocário.

Figura 10. Modelo de reparo de quebras em fitas duplas (DSB). A extremidade 3`OH invade o duplex homólogo atuando como primer para a síntese de DNA. As duas junções de Holliday formadas são resolvidas pela clivagem endonucletídica gerando produtos recombinantes e não-recombinantes.

Figura 11: Origem de linhagens diplóides heterozigotos (-/+ and +/-) e homozigotos (+/+) após crossing-over mitótico entre o gene *paba* e o centrômero. * Os diplóides homozigotos (-/-) não são selecionados MM (modificado de Pires e Zucchi 1994).

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