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METHOD PAPER

Limitation of nitrogen source facilitated the production of nonmeiotic recombinants in *Aspergillus nidulans*

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Abstract

Aspergillus nidulans is a fungal model organism extensively used in genetic approaches. It may reproduce sexually and asexually, with a well-defined parasexual cycle. The current paper demonstrates that the limitation of nitrogen source facilitates the production of *A. nidulans*'s nonmeiotic recombinants directly from heterokaryons, without the recovery of the diploid phase. Heterokaryons formed between master strains were inoculated in sodium nitrate-low (basal medium [BM]) and sodium nitrate-rich media (minimal medium [MM]). All mitotic segregants produced by the heterokaryons were tested for their mitotic stability in the presence of benomyl, the haploidizing agent. Only mitotically stable haploid segregants were selected for subsequent analysis. Phenotypic analyses of such haploids favored the characterization of nonmeiotic recombinants. As the number of such recombinants was higher in BM than in MM, nitrogen limitation may have facilitated the isolation of nonmeiotic recombinants from heterokaryons by stimulating nuclear fusion still inside the heterokaryotic mycelium as a survival strategy.

KEYWORDS

mitotic recombination, basal medium, heterokaryosis, parameiosis

The *Aspergillus* genus comprises approximately 350 species of filamentous fungi and represents one of the most widely studied genera of fungi. Species of the genus include opportunistic human pathogens (*A. fumigates*, *A. terreus*), aflatoxin-producing plant pathogens (*A. flavus*, *A. parasiticus*), species of industrial interest (*A. niger, A. oryzae*), and a species extremely important for genetic approaches, namely *A. nidulans*, which have contributed significantly to our understanding of eukaryotic molecular and cell biology. *A. nidulans* features sexual and asexual reproduction, coupled to a well-defined parasexual cycle, which starts through heterokaryon formation [1-3].

Hyphal anastomosis, a common process in filamentous fungi, occurs when two mycelia of the same species come into contact. A heterokaryotic mycelium is formed when fusing hyphae carry genetically distinct nuclei. Two distinct haploid nuclei may fuse inside the heterokaryotic mycelium to give rise to a heterozygous diploid nucleus [4,5]. Mitotic crossing-over may occur during the divisions of diploid nuclei and recombinant diploid nuclei may be produced. Furthermore, errors may occur during mitotic divisions of the diploid nucleus, causing sequential losses of chromosomes previously present in the two copies, and resulting in the production of recombinant haploid nuclei. The sequence of events that yield such recombinant nuclei constitutes the

parasexual cycle that provides an effective pathway to increase genetic variability in fungi, especially those with no sexual cycle [3,6,7].

Alternatively, an atypical parasexual cycle, named parameiosis, has been described in some filamentous fungi, including *Aspergillus niger*, *Colletotrichum sublineolum*, and *Beauveria bassiana,* that produces haploid recombinants directly from heterokaryons [8-10]. In this process, very unstable diploid nuclei are formed inside the heterokaryotic mycelium where they undergo mitotic recombination and haploidization, causing nonmeiotic recombinants to emerge from the heterokaryotic mycelium as vigorous mitotic sectors. Although the diploid phase is actually produced in parameiosis, diploids may neither be observed nor isolated [8-10]. In contrast, in the parasexual cycle [3], the diploid nuclei produced within the heterokaryotic hyphae emerge from the heterokaryon as visible sectors. Such diploids may be isolated and maintained indefinitely in the appropriate culture media, where they may also yield haploid recombinants [3-5].

In *Aspergillus* spp, environmental factors including temperature, availability of an air-surface interface and nutrients affect sexual and asexual development. Additionally, nutritional factors such as carbon source and nitrogen source may affect mycotoxin production and morphological differentiation in the genus [11,12]. As a nitrogen source, nitrate has been shown to repress the synthesis of aflatoxin intermediates in *A. parasiticus* while enhancing sterigmatocystin production in *A. nidulans* [13,14]. In *Fusarium oxysporum*, the pairing of vegetative incompatible strains under stressful environmental conditions and under carbon starvation and nitrogen limitation has been reported to produce, respectively, nonmeiotic recombinants and viable heterokaryotic cells [15,16]. In the current study, limitation of nitrogen source facilitated the production of *A. nidulans*'s nonmeiotic recombinants directly from heterokaryons and parameiosis has been associated with the production of such recombinants.

Heterokaryons and nonmeiotic recombinants were inoculated in different culture media: (a) sodium nitrate-low medium (basal medium, BM): 1,000 ml distilled H_2O; 10 g sucrose; 2 g $NaNO_3$; 1 g KH_2PO_4; 0.5 g $MgSO_4 \cdot 7\ H_2O$; 0.5 g KCl; 10 mg $FeSO_4 \cdot 7\ H_2O$; 15 g agar; 0.2 ml trace element solution (95 ml distilled H_2O; 5 g citric acid; 1 g $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$; 0.25 g $CuSO_4 \cdot 5\ H_2O$; 50 mg $MnSO_4 \cdot H_2O$; 50 mg H_3BO_3; and 50 mg $Na_2MoO_4 \cdot 2\ H_2O$); (b) sodium nitrate-rich medium (minimal medium, MM): 1,000 ml distilled H_2O; 10 g glucose, 6 g $NaNO_3$; 0,52 g KCl; 1.52 g KH_2PO_4; 0.52 g $MgSO_4.7\ H_2O$; 2 mg $FeSO_4.7\ H_2O$; 1 mg $ZnSO_4 \cdot 7H_2O$; 1 mg $CuSO_4 \cdot 5\ H_2O$; 15 g agar; (c) selective medium (SM) consisting of MM supplemented with nutritional requirements of the crossing strains with the omission of one of them, in each type of medium; (d) complete medium (CM) [7]. *A. nidulans*'s master strains used to form heterokaryons and the nonmeiotic recombinants obtained from such heterokaryons are described in Table S1.

Approximately 2×10^7 conidia/ml of two master (or parental) strains genetically complementary for nutritional and conidia color markers were coinoculated in liquid MM + 2.0% liquid CM to obtain the heterokaryons. Heterokaryons are prototrophic mosaic colonies consisting of conidia of different colors derived from the two crossed strains. The heterokaryons, obtained after incubation for three days at 37°C were then inoculated into two sets of ten Petri dishes, each set containing BM or MM. When necessary, the culture medium was supplemented with a nutritional requirement common to both strains used to form heterokaryons. Plates were incubated for 7–12 days at 37°C. After this period, the plates were inspected for the presence of vigorous mitotic sectors originating directly from heterokaryons. Sectors were purified in CM and they were tested for their mitotic stability in the presence of benomyl (0.2 μg/ml), the haploidizing agent. Only mitotically stable segregants growing in CM + benomyl were considered haploid and selected for phenotypic analysis in differential SM.

All nonmeiotic recombinants were sexually crossed with master strains, so that hybrid cleistothecia were obtained from heterokaryons after a 21-day incubation period, in sealed plates containing solid SM or MM, according to requirements of crossed strains. The meiotic segregants obtained from hybrid or self-fertilized cleistothecia were replicated on a series of solid-agar plates containing differential SM for their phenotypic characterization. Standard χ^2 was used to test the expected 1:1 ratio for allelic genes.

Some nonmeiotic recombinants were inoculated in liquid CM for genomic DNA extraction and Random amplification of polymorphic DNA–polymerase chain reaction (RAPD-PCR) amplification [17]. Primers (Operon Technologies) OPA-19 (5′-CAAACGTCGG-3′), OPA-20 (5′-GTTGCGATCC-3′), OPE-10 (5′-CACCAGGTGA-3′), OPE-20 (5′-AACGGTGACC-3′), OPW-02 (5′-ACCCCGCCAA-3′), OPW-03 (5′-GTCCGGAGTG-3′), OPW-04 (5′-CAG AAGCGGA-3′), OPW-07 (5′-CTGGACGTCA-3′), OPW-10 (5′-TCGCATCCCT-3′), and OPX-20 (5′-CCCAGCTAGA-3′) were used for RAPD analysis.

Heterokaryons inoculated in MM and BM produced several mitotic sectors (or mitotic segregants) with the same or distinct phenotypes to the parental crossed strains. Mitotic sectors were isolated, purified in CM and tested for their mitotic stability in CM + benomyl. Three types of segregants were identified in this test: diploids, aneuploids, and stable haploids (Figure 1a,b). The diploids recovered were produced by the fusion of two parental haploid nuclei in the parasexual

cycle, whereas aneuploids were produced when the haploidization of the diploid nuclei was incomplete. Such aneuploids may be produced in the parasexual cycle and in parameiosis. Although in the presence of benomyl the diploid segregants originated new mitotic segregants (Figure 1a), the aneuploid ones produced colonies with irregular edges and abnormal growth when compared to the master strains (Figure 1b, left). In contrast, the mitotically

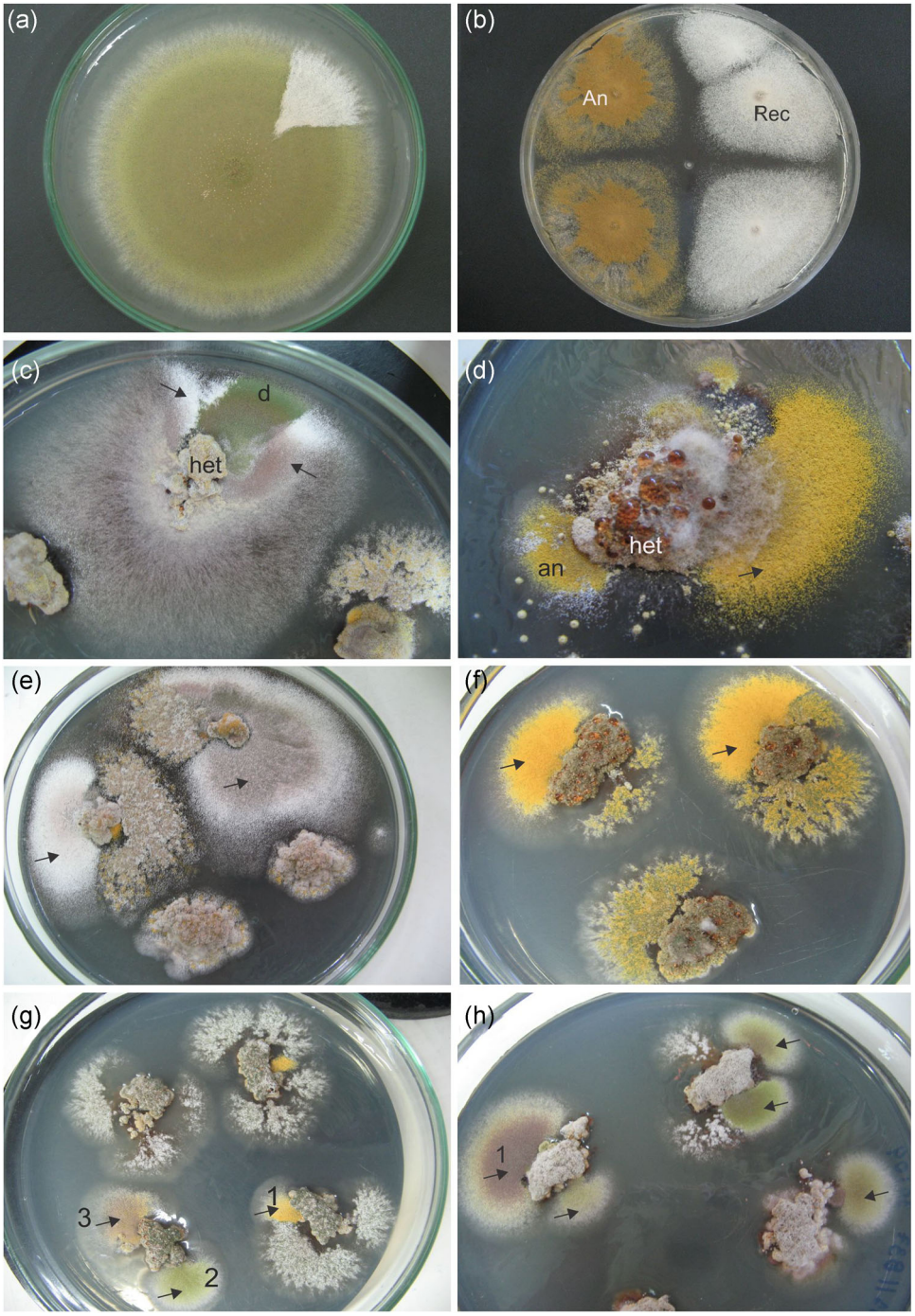

FIGURE 1 Diploid formed by master strains A757 and UT448 (a), aneuploid segregant derived from diploid A757//UT448 (b, left) and stable haploid segregant (b, right) growing in CM + benomyl. Nonmeiotic recombinants derived from heterokaryons in BM: (c, d) UT448/A757, (e) UT448/A411, (f) A507/A757, (g) A507/B211, and (h) A837/B211. Arrows indicate the haploid nonmeiotic recombinants. The characterization of the mitotic sectors as haploid, diploid and aneuploid was made according to their growth in CM + benomyl. In the presence of benomyl, diploid nuclei produce new mitotic sectors; aneuploids originate colonies with irregular edges and poor growth; haploids remain stable. Heterokaryons were identified by the presence of conidia of the two master stains used to form heterokaryons. BM, basal medium; CM, complete medium; an, aneuploid; d, diploid; het, heterokaryon; rec, nonmeiotic recombinant

TABLE 1 Number of nonmeiotic recombinants obtained in MM and BM

	No. of nonmeiotic recombinants in MM			No. of nonmeiotic recombinants in BM		
Heterokaryons	**i.a.**	**m.c.o.**	**Total**	**i.a.**	**m.c.o.**	**Total**
UT448/A757	0	02	02	21	03	24
A507/A757	04	0	04	23	0	23
A411/UT448	0	0	0	0	04	04
A411/A219	03	04	07	04	02	06
A507/B211	0	05	05	02	03	05
A507/A837	01	0	01	25	0	25
A837/B211	0	11	11	03	20	23
A837/A757	30	0	30	50	0	50
Total	38	22	60*	128	32	160*

Note: Total is the total number of recombinants.
Abbreviations: BM, basal medium; MM, minimal medium; i.a., independent assortment; m.c.o., mitotic crossing-over.
*Statistically significant $p < .05$ (data were analyzed using a negative binomial model).

stable segregants growing in CM + benomyl (Figure 1b, right) were considered haploids and were submitted to phenotypic analysis. Parental and recombinant phenotypes were identified (Table S2). Haploid recombinants were named nonmeiotic recombinants and were obtained only by parameiosis. As a whole, the results from Table S2 and Figure 1c suggest that both the parasexual cycle and parameiosis may occur simultaneously in a single heterokaryotic mycelium.

Nonmeiotic recombinants, harboring the nutritional markers from strains used to form heterokaryons, emerged directly from the heterokaryons in BM and MM. However, the number of such recombinants in BM was higher than that in MM (Table 1). Although recombinants originated by mitotic crossing-over or independent assortment of the parental chromosomes, the latter's frequencies were the highest in most of the heterokaryons. Exceptionally, the heterokaryon formed by master strains A837 and B211, both bearing *uvsH* mutation, produced a large number of intergenic mitotic recombinants in MM and BM (Table 1). The above was indeed expected from *uvsH/uvsH* heterokaryon, as *uvsH* mutation causes high indexes of intergenic mitotic recombination in *A. nidulans* [18,19].

Nonmeiotic recombinants exhibiting parental or recombinant phenotypes for the conidia color markers (Table S1) are shown in Figure 1c–h. The heterokaryon formed by A507 and B211 strains, respectively, with green (*y*+; *w*+; *cha*+) and white (*y*; *w*; *cha*) conidia, produced recombinants with yellow (*y*; *w*+; *cha*+; Figure $1g_1$), chartreuse (*y*+; *w*+; *cha*; Figure $1g_2$) and yellow-chartreuse (*y*; *w*+; *cha*; Figure $1g_3$) conidia. In addition, the heterokaryon formed by A837 and B211 strains, with chartreuse (*y*+; *w*+; *cha*) and white (*y*; *w*; *cha*) conidia, respectively, produced recombinants with yellow-chartreuse (*y*; *w*+; *cha*) conidia (Figure $1h_1$).

Four recombinants derived from A837/B211 heterokaryon were characterized by the RAPD technique. Primers OPW-07 (5′-CTGGACGTCA-3′) and OPW-03 (5′-GTCCGGAGTG-3′) generated DNA fragments of

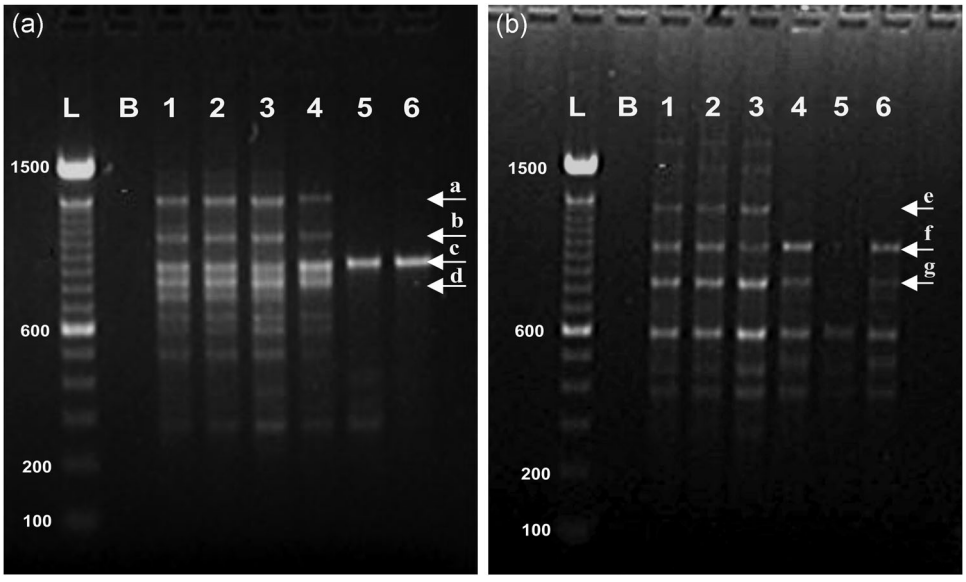

FIGURE 2 RAPD profiles of parental strains A837 (line 1) and B211 (line 2), and recombinants R13, R14, R15, and R16 (lines 3, 4, 5, and 6) in agarose gel using primers OPW-07 (a) and OPW-03 (b). Arrows indicate polymorphic bands of a, 1,500; b, 1,200; c, 950; and d, 850 bp obtained with primer OPW-07; bands of e, 1,400; f, 1,100; and g, 850 bp obtained with primer OPW-03. L and B, molecular markers (base pairs [bp]) and negative control, respectively

approximately 1,500, 1,200, 950, and 850 bp by OPW-07, and 1,400, 1,100, and 850 bp by OPW-03 (Figure 2a,b). The RAPD markers obtained with primer OPW-07 were present in A837 and B211 parental strains, and in recombinants R13 and R14, but were missing in recombinants R15 and R16 (Figure 2a). In the RAPD profile obtained with primer OPW-03, a band of 1.400 bp, observed in A837, B211, and R13 profiles, was not identified in R14, R15, and R16. Furthermore, bands of 850 and 1,100 bp found in A837, B211, R13, R14, and R16 profiles were not detected in the R15 RAPD profile (Figure 2b; For clarity, only the results obtained with primers OPW-03 and OPW-07 are shown).

Nitrogen limitation was previously reported to induce the dimorphic transition to pseudohyphal growth in *Saccharomyces cerevisiae* and to increase the rate of conidial anastomosis tubes fusions in *F. oxyporum*, resulting in the formation of viable heterokaryotic hyphae [15,20]. In *Penicillium griseofulvum*, nitrogen starvation was associated with a rapid increase in intracellular proteinase activity. This response was considered adaptive by the authors, permitting fungal survival when assimilating nitrogen was absent from the external environment [21]. As genetic exchanges between compatible fungal strains cause them to update their genomes [5], in our analysis, the limited access to nitrogen in BM may have stimulated the fusion of parental nuclei inside the heterokaryon as a survival strategy and facilitated the emergence of new recombinant genotypes, more adapted to the limited nutrient availability. As previously observed in *A. niger* and *C. sublineolum* [8,22], the nonmeiotic recombinants obtained in our assay have probably originated from highly unstable diploid nuclei that, posterior to mitotic recombination, rapidly returned to the haploid condition, still in the heterokaryotic hyphal stage, and before conidia formation [8-10,22].

When submitted to sexual crosses, most mitotic recombinants exhibited the Mendelian segregation of genetic markers, demonstrating their meiotic stability. On the contrary, cleistothecia containing a reduced number of viable ascospores were obtained in the R7 × A507 cross which may be explained by R7 recombinant's aneuploid condition (Table S3).

Parasexual recombinants, produced in the parasexual cycle, resulting from the haploidization of diploid nuclei, which may be isolated and maintained indefinitely in the appropriate culture media [5]. On the contrary, nonmeiotic recombinants were obtained in the current study only by the parameiotic process, which does not allow the recovery of the diploid phase (Table S4). Results in the current study strongly suggest that the limitation of nitrogen source facilitates obtaining nonmeiotic recombinants in *A. nidulan.*

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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