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PRISCILA AYUMI SYBUIA

RECOMBINAÇÃO PARASSEXUAL EM *Aspergillus nidulans* E *Macrophomina pseudophaseolina*, E REAÇÕES DE COMPATIBILIDADE VEGETATIVA EM *M. euphorbiicola*

Maringá
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutora em Ciências Biológicas.

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BIOGRAFIA

Priscila Ayumi Sybuia nasceu em Maringá/PR em 25 de agosto de 1993. Filha de Mauricio Fumio Sybuia e Fabiana de Oliveira Silva Sybuia. Possui graduação em Tecnologia em Biotecnologia (2015) pela Universidade Estadual de Maringá (UEM). Neste período, integrou o Laboratório de Biotecnologia Microbiana (LBIOMIC) onde participou de projetos de avaliação de atividade antagônica e produção de metabólitos secundários por fungos endofíticos. De 2016 a 2018, cursou o mestrado no Programa de Pós-graduação em Ciências Biológicas (Área de concentração – Biologia Celular e Molecular), fazendo parte do laboratório Bioquímica de Microrganismos e Alimentos (LBM), onde investigou a biorremediação de fármacos por enzimas produzidas por *Trametes* sp. Em 2019, iniciou o doutorado no mesmo curso de pós-graduação. No Laboratório de Genética de Microrganismos (LGM), investigou a ocorrência de parassexualidade em fungos ascomicetos. Em 2020, concluiu o curso de graduação em Ciências Biológicas (Universidade Estadual de Maringá). Possui experiência nas áreas de Biorremediação, Bioquímica de Microrganismos, Genética de Microrganismos e Biologia Molecular.

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“Ainda que eu ande pelo vale da sombra da morte, não temerei mal algum, porque tu estás comigo”

Salmo 23:4

APRESENTAÇÃO

Esta tese, intitulada **Recombinação parassexual em *Aspergillus nidulans* e *Macrophomina pseudophaseolina*, e reações de compatibilidade vegetativa em *M. euphorbiicola***, é composta por dois artigos científicos: Limitation of nitrogen source facilitated the production of nonmeiotic recombinants in *Aspergillus nidulans* e Parasexual recombination in *Macrophomina pseudophaseolina* and vegetative compatibility reactions in *M. euphorbiicola*. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, os artigos foram redigidos de acordo com as normas das revistas Journal of Basic Microbiology e European Journal of Plant Pathology.

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SUMÁRIO

RESUMO GERAL

O ciclo parassexual, observado primeiramente no ascomiceto *Aspergillus nidulans*, e posteriormente em diversas espécies de fungos filamentosos, é considerado uma alternativa ao ciclo sexual, uma vez que recombinantes parassexuais são produzidos sem a ocorrência do processo meiótico. O ciclo parassexual tem início com a formação do micélio heterocariótico, que resulta da fusão de hifas de dois isolados vegetativamente compatíveis, sendo constituído por dois núcleos haploides, geneticamente distintos. Tais núcleos podem eventualmente se fundir, dentro do micélio heterocariótico, originando um núcleo diploide heterozigoto. Estes núcleos diploides podem espontaneamente produzir recombinantes diploides, por meio de *crossing-over* mitótico, e recombinantes haploides, pelo processo de não-disjunção mitótica, no qual a perda aleatória de um membro de cada par cromossômico resulta na condição haploide do núcleo. Em um ciclo parassexual atípico, denominado parameiose, os recombinantes não-meióticos emergem diretamente do micélio heterocariótico, como setores mitóticos vigorosos. Neste processo, o núcleo diploide é formado dentro do micélio heterocariótico, onde sofre recombinação mitótica e haploidização. Embora a fase diploide seja produzida na parameiose, os diploides não podem ser observados ou isolados. O fungo filamentoso *Aspergillus nidulans* é amplamente utilizado em abordagens genéticas e apresenta-se como um excelente sistema para o estudo da recombinação mitótica. Isso se justifica pelo fato deste microrganismo passar grande parte do seu ciclo celular em G2, fase em que os cromossomos se encontram duplicados, favorecendo a ocorrência da recombinação mitótica. Em *Aspergillus* spp., temperatura, disponibilidade de oxigênio e fatores nutricionais afetam tanto o desenvolvimento assexual quanto o sexual. A concentração de nitrogênio mostrou-se capaz de reprimir a produção de intermediários da síntese de aflatoxinas em *A. parasiticus*, e estimular a produção de esterigmatocistina em *A. nidulans*. Com base nestas observações, o objetivo do primeiro estudo que compõe esta Tese de Doutorado foi avaliar a influência da limitação da fonte de nitrogênio na produção de recombinantes não-meióticos em *Aspergillus nidulans*. Para atingir os objetivos propostos, linhagens mestras de *A. nidulans*, geneticamente complementares para marcadores nutricionais e de coloração dos conídios, foram coinoculadas para a obtenção de heterocários. Os heterocários formados foram inoculados em dois tipos de meios de cultura: um com baixa concentração de nitrato de sódio (Meio Basal [MB]), e outro, com alta concentração de nitrato de sódio (Meio Mínimo [MM]). Após 12 dias de incubação

a 37 °C, as placas foram inspecionadas quanto à presença de setores mitóticos vigorosos, originados dos heterocários. Os setores que se apresentaram estáveis mitoticamente foram considerados haploides e analisados fenotipicamente. Estas análises permitiram a identificação de recombinantes não-meióticos, os quais foram caracterizados como parameióticos, uma vez que emergiram diretamente dos heterocários. Nestas análises foi também possível verificar que o número de recombinantes não-meióticos obtido em MB (baixa concentração de nitrato de sódio) foi maior do que aquele obtido em MM (alta concentração de nitrato de sódio). A baixa concentração de nitrogênio em MB pode ter sido responsável pela origem de um maior número de recombinantes não-meióticos, do que aquele observado em MM. A produção de recombinantes seria uma tentativa de originar novos genótipos, melhor adaptados às condições limitadas de nutrientes disponíveis no meio de cultura. Alguns recombinantes não-meióticos, quando analisados pela técnica do RAPD, produziram padrões de bandas de RAPD que possibilitaram diferenciá-los das linhagens paternais, comprovando seus genótipos recombinantes. Os resultados deste estudo sugerem, portanto, que a limitação de nitrogênio facilita a obtenção de recombinantes não-meióticos em *A. nidulans.*

O segundo estudo desta Tese de Doutorado teve como objetivos estudar: a) a diversidade das reações de compatibilidade vegetativa em isolados brasileiros de *M. pseudophaseolina* e *M. euphorbiicola*, b) a variabilidade genética dos isolados utilizados, e c) a ocorrência de recombinação parassexual em isolados de *M. pseudophaseolina.* O gênero *Macrophomina* é constituído por fungos fitopatogênicos responsáveis pela doença "podridão de carvão" em diversas espécies de plantas. Embora as espécies de *Macrophomina* não apresentem ciclo sexual conhecido, alta variabilidade genética, morfológica e patogênica tem sido observada nestas espécies. Mutantes incapazes de utilizar o nitrato como fonte de nitrogênio (*nit*) dos isolados de *M. pseudophaseolina* e *M. euphorbiicola* foram obtidos e utilizados para a formação de heterocários intra- e inter-isolados. Nos cruzamentos intra-isolados, os isolados 2, 5 (*M. pseudophaseolina*) e 28 (*M. euphorbiicola*) não formaram heterocários. Nas análises de anastomoses de hifas, tais isolados produziram menor número de fusões de hifas/mm^2 em relação aos isolados HSC (*heterokaryon self-compatible*), os quais são capazes de formar heterocários em cruzamentos intra-isolado. Desta forma, os isolados 2, 5 e 28 foram caracterizados como mutantes HSI (*heterokaryon self-incompatible*), os quais são deficientes para o processo de anastomose de hifas, sendo incapazes de formar heterocários. Os pareamentos inter-isolados, realizados entre mutantes *nit* dos 25 isolados HSC de *M. pseudophaseolina*

permitiram o agrupamento dos mesmos em 18 grupos de compatibilidade vegetativa (VCG) distintos: 13 VCGs formados por apenas um isolado, três VCGs compostos por dois isolados, um constituído por três isolados, e um contendo quatro isolados. Correlações entre VCGs e origem geográfica dos isolados não foram observadas, uma vez que isolados provenientes de um mesmo município foram alocados em distintos VCGs. Em *M. euphorbiicola*, os dois isolados HSC (29 e 30) não formaram heterocário viável entre si, sendo, portanto, alocados em diferentes VCG. Adicionalmente, os isolados de *M. euphorbiicola* também não formaram heterocários viáveis com os isolados de *M. pseudophaseolina.* Heterocários formados entre mutantes *nit* complementares do isolado 14, e entre mutantes *nit* complementares dos isolados 4 e 6 de *M. pseudophaseolina*, produziram diploides prototróficos, nomeados D14//14 e D4//6, respectivamente. Quando cultivados em MB + $NaNO_3$, ambos os diploides produziram, espontaneamente, segregantes prototróficos recombinantes, que se apresentaram estáveis em presença de benlate, confirmando sua condição haploide. A variabilidade genética dos 27 isolados de *M. pseudophaseolina* e dos 3 de *M. euphorbiicola* foi analisada pela técnica do RAPD, com a utilização de nove primers, que permitiram a construção de um dendrograma. Um total de 136 bandas polimórficas foram obtidas, as quais permitiram agrupar os isolados em quatro grupos de RAPD (I a IV) e dois subgrupos (Ia e Ib). Não foi possível identificar correlação significativa entre genótipos, localização geográfica e hospedeiros dos isolados, uma vez que isolados provenientes de diferentes localidades ou hospedeiros formaram um mesmo grupo de RAPD e, de forma contrária, isolados provenientes de um mesmo município, foram alocados em grupos diferentes (Ib e II, respectivamente). Embora não tenha sido possível caracterizar o ciclo parassexual em *M. euphorbiicola* devido ao reduzido número de isolados analisados, nossos resultados demonstram pela primeira vez, a ocorrência do ciclo parassexual em *M. pseudophaseolina*, com a produção de recombinantes mitóticos e sugerem que a recombinação parassexual pode desempenhar um papel importante na variabilidade genética desta espécie, sendo em parte responsável pelo surgimento de novos patótipos.

Palavras-chave: Ciclo parassexual, parameiose, recombinação mitótica, mutantes *nit*, compatibilidade vegetativa, RAPD.

GENERAL ABSTRACT

The parasexual cycle, first observed in the ascomycete *Aspergillus nidulans*, and subsequently in several species of filamentous fungi, is considered an alternative to the sexual cycle, since parasexual recombinants are produced without the occurrence of meiotic process. Parasexual cycle begins with the heterokaryotic mycelium formation, that results from hyphal fusion of two vegetatively compatible isolates, consisting of two genetically distinct haploid nuclei. Such nuclei may eventually fuse within the heterokaryotic mycelium, giving rise to a heterozygous diploid nucleus. These diploid nuclei can spontaneously produce diploid recombinants, through mitotic crossing-over, and haploid recombinants, by the process of mitotic non-disjunction, on which the random loss of one member of each chromosome pair results in haploid condition of the nucleus. In an atypical parasexual cycle, named parameiosis, non-meiotic recombinants emerge directly from the heterokaryotic mycelium as vigorous mitotic sectors. In this process, diploid nuclei are formed inside the heterokaryotic mycelium where they undergo mitotic recombination and haploidization. Although the diploid phase is actually produced in parameiosis, diploids may neither be observed nor isolated. The filamentous fungus *Aspergillus nidulans* is extensively used in genetic approaches and is an excellent system to the mitotic recombination study. This is due to the fact that this microorganism spends much of its cell cycle in G2, a phase in wich the chromosomes are duplicated, favoring the occurrence of mitotic recombination. In *Aspergillus* spp., temperature, oxygen availability, and nutritional factors affect both asexual and sexual development. Nitrogen concentration was able to repress the aflatoxin synthesis intermediates production in *A. parasiticus* and to stimulate sterigmatocystin production in *A. nidulans.* Based on these observations, the aim of the first study that comprises this Doctoral Thesis was to evaluate the influence of nitrogen source limitation on the production of nonmeiotic recombinants in *Aspergillus nidulans.* To achieve the proposed objectives, master strains of *A. nidulans*, genetically complementary for nutritional and conidia color markers, were coinoculated to obtain heterokaryons. The heterokaryons formed were inoculated in two types of culture medium: sodium nitrate-low (basal medium [BM]) and sodium nitrate-rich media (minimal medium [MM]). After 12 days of incubation at 37 ^{o}C, plates were inspected for the presence of vigorous mitotic sectors originating directly from heterokaryons. Sectors presented as mitotically stable were considered haploid and phenotypically analysed. These analyses allowed the identification of nonmeiotic

recombinants, and characterized them as parameiotics, since they emerged directly from heterokaryons. In these analyses, it was also possible to verify that the number of nonmeiotic recombinants obtained in BM (sodium nitrate-low) was higher than that in MM (sodium nitrate-rich). The low concentration of nitrogen in BM may be responsible for a higher number of nonmeiotic recombinants´ origin, than the observed in MM. The production of recombinants may be an attempt to originate new genotypes, better adapted to the nutrient-limited conditions in culture medium. Some nonmeiotic recombinants, when analysed by RAPD technique, produced RAPD band patterns that allowed the differentiation of parental strains, proving their recombinant genotype. The results of this study suggest therefore that nitrogen limitation facilitates the obtainment of nonmeiotic recombinants in *A. nidulans.*

The second study that comprises this Doctoral Thesis aims to evaluate a) the diversity of vegetative compatibility reactions of the Brazilian isolates of *M. pseudophaseolina* and *M. euphorbiicola*, b) genetic variability of the isolates utilized, and c) the occurrence of parasexual recombination in *M. pseudophaseolina* isolates. The genus *Macrophomina* is composed of phytopathogenic fungi responsible for the charcoal rot disease in several plant species. Although *Macrophomina* species have no known sexual stage, a high genetic, morphological, and pathogenic variability has been observed in these species. Mutants unable to use sodium nitrate as a nitrogen source (*nit* mutants) were obtained from *M. pseudophaseolina* and *M. euphorbiicola* isolates and used in the formation of intra- and- inter-isolates heterokaryons. In intra-isolates crosses, isolates 2, 5 (*M. pseudophaseolina*), and 28 (*M. euphorbiicola*) did not form heterokaryons. In hyphal anastomosis analyses, such isolates produced a smaller number of hyphal fusions/mm^2 in relation to HSC (*heterokaryon self-compatible*) isolates, which are capable of forming heterokaryons in intra-isolates crossing. Thus, isolates 2, 5, and 28 were characterized as HSI (*heterokaryon self-incompatible*) mutants, which are defective for hyphal anastomosis process. Inter-isolates pairing performed between *nit* mutants of the 25 *M. pseudophaseolina* HSC isolates allowed their grouping into 18 distinct vegetative compatibility groups (VCG): 13 VCGs consisted of a single isolate, three consisted of two isolates, one consisted of three isolates, and one contained four isolates. Correlations between VCG and isolates´ geographic origin were not found since most isolates derived from the same localities were grouped in distinct VCGs. In *M. euphorbiicola,* the HSC isolates (29 and 30) did not form viable heterokaryon among themselves and therefore allocated in different VCG. Additionally, *M. euphorbiicola* isolates did not form viable

heterokaryon with *M. pseudophaseolina* isolates. Heterokaryons formed between complementary *nit* mutants of isolate 14, and between complementary *nit* mutants of isolates 4 and 6 of *M. pseudophaseolina*, produced prototrophic diploids, named D14//14 and D4//6, respectively. When cultivated in BM + $NaNO_3$, both diploids spontaneously produced recombinant prototrophic segregants, stable in the presence of benlate, confirming their haploid condition. The genetic variability of the 27 *M. pseudophaseolina* and the 3 *M. euphorbiicola* isolates was analysed using nine primers in the RAPD technique, which allowed the construction of a dendrogram. A total of 136 polymorphic bands were obtained and allowed grouping the isolates in four RAPD groups (I to IV) and two subgroups (Ia and Ib). It was not possible to identify significant correlations between genotype, geographic location, and hosts, since isolates derived from distinct localities or hosts formed the same RAPD group and, conversely, isolates from the same municipality were allocated into different groups (Ib and II, respectively). Although it was not possible to characterize the parasexual cycle in *M. euphorbiicola*, due to the reduced number of isolates analysed, our results demonstrate for the first time the occurrence of the parasexual cycle in *M. pseudophaseolina*, with the production of mitotic recombinants, and suggest that parasexual recombination may play an important role in genetic variability of this species, being partly responsible for the emergence of new pathotypes.

Keywords: Parasexual cycle, parameiosis, mitotic recombination, *nit* mutants, vegetative compatibility, RAPD.

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.

1 INTRODUCTION

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.

2 MATERIALS AND METHODS

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 $NaNO_3$; 1g 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 $MgSO_4 \cdot 7\ H_2O$; 2 mg $FeSO_4 \cdot 7\ H_2O$; 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 $\chi 2$ 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′-CAGAAGCGGA-3′), OPW-07 (5′-CTGGACGTCA-3′), OPW-10 (5′-TCGCATCCCT-3′), and OPX-20 (5′-CCCAGCTAGA-3′) were used for RAPD analysis.

3 RESULTS AND DISCUSSION

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 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 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 R5 × A507 cross which may be explained by R5 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. nidulans*.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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Table 1. Number of non-meiotic recombinants obtained in MM and BM.

Heterokaryons	Nº. of non-meiotic recombinants in MM			Nº. of non-meiotic recombinants in BM		
	i.a.[a]	m.c.o.[b]	Total	i.a.[a]	m.c.o.[b]	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 analysed using a negative binomial model).

Table S1. Genotypes and origin of *Aspergillus nidulans* strains and non-meiotic recombinants (R1-R16).

Strains	Genotypes	Origin
A757	*yA2*; *methA17*; *pyroA4*.	FGSC[a]
A411	*yA2, proA1, pabaA1,*	FGSC[a]
A837	*chaA1; pabaA1*; *uvsH77, pyroA4; choA1*	FGSC[a]
A219	*biA1*; *methG*	FGSC[a]
A507	*FpaB37*, *SulA*1, *anA1, pabaA1*	FGSC[a]
UT448	*wA3, riboA1, pabaA124, biA1*; *AcrA1*	UUT[b]
B211	*wA3; yA2*; *AcrA1, methA17; uvsH77, pyroA4; chaA1*	LGM[c]
R1[d]	*yA2*	UT448/A757*
R2	*wA3*	UT448/A757*
R3	*yA2, uvsH77, pyroA4, chaA1*	A837/B211*
R4	*wA3, uvsH77, pyroA4, chaA1*	A837/B211*
R5	*yA2, pyroA4*	A507/A757*
R6	*biA1*	A411/A219*
R7	*pabaA1*	A837/A507*
R8	*yA2, pabaA1*	A411/UT448**
R9	*yA2*, *pyroA4*	A837/A757**
R10	*wA3*, *biA1*, *pyroA4*	A507/B211**
R11	*pyroA4*	UT448/A757**
R12	*wA3, biA1*	UT448/A757**
R13	*yA2; uvsH77, pyroA4; chaA1*	A837/B211*
R14	*wA3, uvsH77, pyroA4; chaA1*	A837/B211**
R15	*yA2, uvsH77, pyroA4; chaA1*	A837/B211*
R16	*wA3, uvsH77, pyroA4; chaA1*	A837/B211*

Mutant alleles have the following phenotypes: *pro*, *an*, *ribo*, *paba*, *bi*, *meth*, *pyro*, and *cho*: requirements for proline, aneurine, riboflavine, paraminobenzoic acid, biotin, methionine, pyridoxine, and choline respectively. *y*, *w*, *cha* and *fw*: yellow, white, chartreuse and fawn conidial color, respectively. *Acr*, *Fpa*, *Sul* determine resistance to acriflavine, *p*-fluorophenylalanine and sulphanilamide, respectively. *uvs*: sensibility to UV light. [a] Fungal Genetic Stock Center, Kansas, USA. [b] collection of microorganisms of Utrecht University, The Netherlands. [c] Laboratory of Genetic of Microrganisms, Universidade Estadual de Maringá, Paraná, Brazil. [d] R1-R16: non-meiotic recombinants. *Heterokaryons inoculated in BM. **Heterokaryons inoculated in MM

Table S2. Mitotic sectors (or segregants) derived from heterokaryons in MM and BM.

Heterokaryons	Mitotic segregants obtained in MM[a]					Mitotic segregants obtained in BM[b]				
	An[c]	D[d]	Haploids		Total	An[c]	D[d]	Haploids		Total
			Pat[e]	Rec[f]				Pat[e]	Rec[f]	
UT448 / A757	16	01	14	02	33	39	05	19	24	87
A507 / A757	17	0	02	04	23	11	04	03	23	41
A411 / UT448	19	0	3	0	22	15	0	16	4	35
A411 / A219	11	01	03	07	22	09	02	18	06	35
A507 / B211	11	0	06	05	22	12	01	03	05	21
A507 / A837	01	0	0	01	02	42	01	0	25	68
A837 / B211	08	0	01	11	20	08	03	07	23	36
A837 / A757	22	0	03	30	55	28	02	04	50	74

[a] minimal medium; [b] basal medium; [c] aneuploids (obtained both in parameiosis and in the parasexual cycle); [d] diploids (obtained in the parasexual cycle); [e] parentals (obtained only in parameiosis); [f] non-meiotic recombinants (obtained only in parameiosis).

Table S3. Meiotic segregation of the genetic markers of chromosomes I, II, and IV in the sexual crosses between non-meiotic recombinants and the master strains A507, A757 or A219.

Sexual Crosses	**Meiotic segregation of genetic markers**				
	an+: *an*	*paba+*: *paba*	*bi+*: *bi*	*meth+*: *meth*	*pyro+: pyro*
A507 x A757[a]	54 : 69	58 : 65	n.a.	71 : 52	70 : 53
R1 x R1 [b]	100 : 0	100 : 0	100 : 0	100 : 0	100 : 0
R2 x R2 [b]	100 : 0	100 : 0	100 : 0	100 : 0	100 : 0
R3 x A507	70 : 54	65 : 59	n.a.	n.a.	76 : 48
R4 x A507	67 : 52	68 : 51	n.a.	n.a.	69 : 50
R5 x A507[c]	41 : 54	42 : 53	n.a.	n.a.	49 : 36
R6 x A757	n.a.	n.a.	52 : 48	48 : 52	52 : 48
R7 x A757	n.a.	66 : 59	n.a.	71 : 54	73 : 52
R8 x A219	n.a.	63 : 60	61 : 62	69 : 54	n.a.
R9 x A507	53 : 50	52 : 51	n.a.	n.a.	51 : 52
R10 x A507	68 : 56	61 : 63	62 : 62	n.a.	n.a.
R11 x A507	51 : 51	51 : 51	n.a.	n.a.	55 : 47
R12 x A507	60 : 46	53 : 53	58 : 48	n.a.	n.a.

[a]control cross, [b]self-fertilized cross, [c] cleistothecia with reduced number of ascospores; n.a. = not analyzed.

Table S4. Processes involved in the production of parasexual and non-meiotic recombinants.

Parasexual recombinants (obtained in the parasexual cycle)	**Non-meiotic recombinants (obtained in parameiosis)**
1- Nuclear fusion of two haploid nuclei inside the heterokaryotic mycelium, resulting in the production of a diploid nucleus.	1- Nuclear fusion of two haploid nuclei inside the heterokaryotic mycelium**,** resulting in the production of a very unstable diploid nucleus.
2-Diploid nuclei emerge from heterokaryotic colony and survive indefinitely in appropriated culture media.	2- Although the diploid phase is actually produced in parameiosis, diploids may neither be observed nor isolated.
3-During the mitotic divisions of the diploid nuclei: a) mitotic crossing-over may occur, resulting in the production of recombinant diploid nuclei, or b) sequential losses of chromosomes previously present in the two copies may occur, resulting in the production of recombinant haploid nuclei.	3-The unstable diploid nucleus undergoes recombination and haploidization still inside the heterokaryotic mycelium, producing non-meiotic recombinants by mitotic crossing over or mitotic non-disjunction.
4- Parasexual recombinants emerge from diploid colonies.	4- Non-meiotic recombinants emerge from the heterokaryotic mycelium.

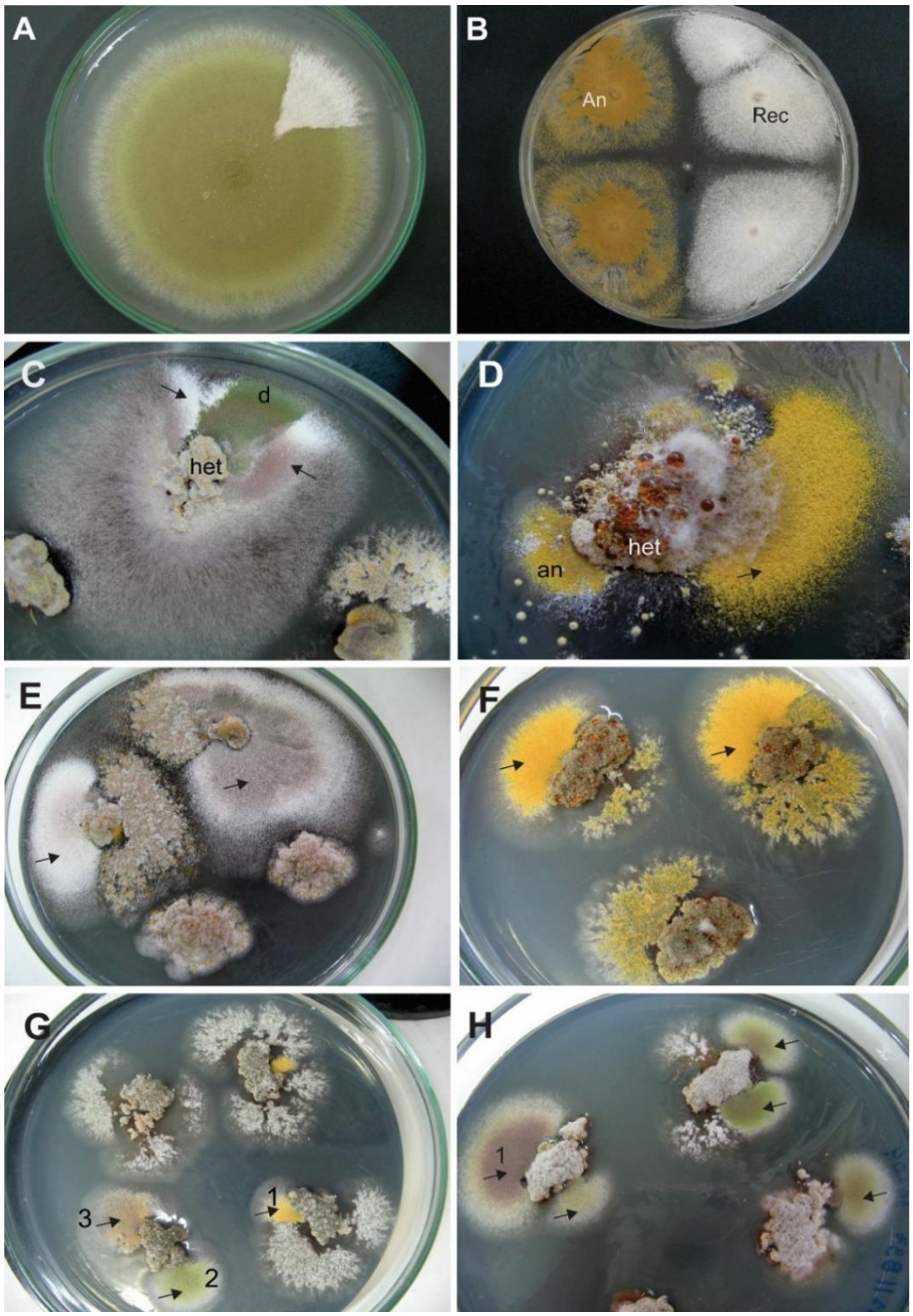

Figure 1. Diploid formed by master strains A757 and UT448 (a), aneuploid segregant derived from diploid A757//UT448 (b, left) and stable haploid segregant (b, right) growing in CM + benomyl. Nonmeiotic recombinants derived from heterokaryons in BM: (c, d) UT448/A757, (e) UT448/A411, (f) A507/A757, (g) 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.

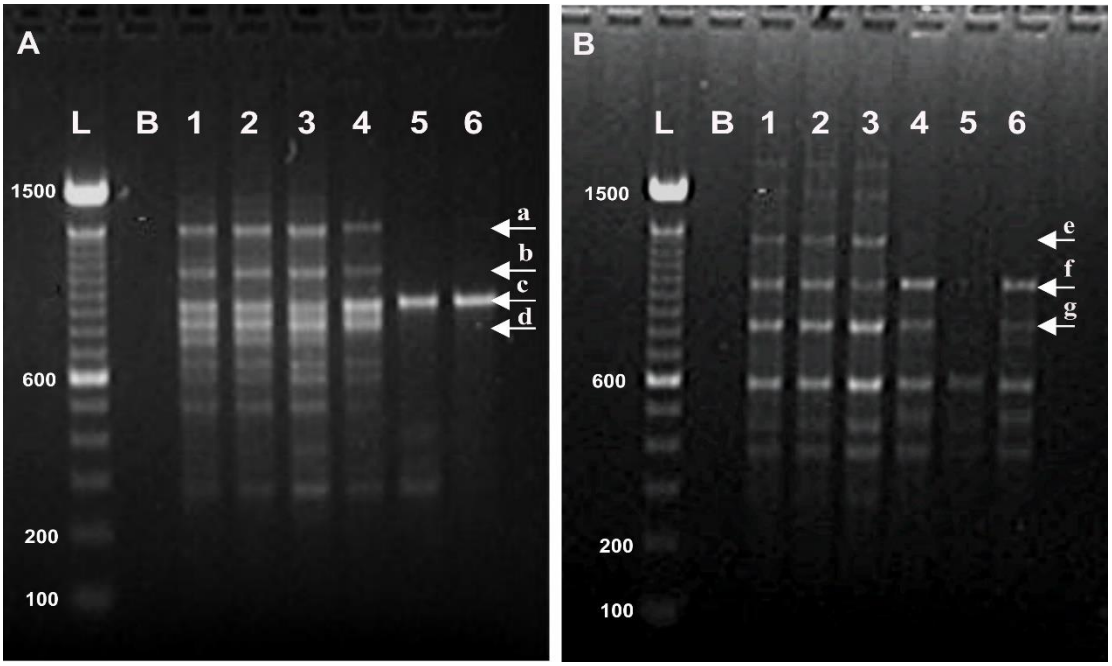

Figure 2. RAPD profiles of parental strains A837 (line 1) and B211 (line 2), and recombinants R13, R14, R15, and R16 (lines 3, 4, 5, and 6) in agarose gel using primers OPW-07 (a) and OPW-03 (b). Arrows indicate polymorphic bands of a, 1,500; b, 1,200; c, 950; and d, 850 bp obtained with primer OPW-07; 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.

Parasexual recombination in *Macrophomina pseudophaseolina* and vegetative compatibility reactions in *M. euphorbiicola*

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ABSTRACT

Charcoal rot is an economically important fungal disease of economically important crops. Several species of the *Macrophomina* genus, such as *Macrophomina pseudophaseolina* and *Macrophomina euphorbiicola*, may act as the causal agents of charcoal rot. The current study evaluates: a) the diversity of vegetative compatibility reactions in Brazilian isolates of *M. pseudophaseolina* (totalling 27) and *M. euphorbiicola* (totalling 3), b) the genetic variability of Brazilian isolates, and c) the occurrence of parasexual recombination in *M. pseudophaseolina* isolates. In complementation tests, 2 and 18 Vegetative Compatibility Groups (VCGs) were identified for the *M. euphorbiicola* and *M. pseudophaseolina* isolates, respectively. Correlations between VCG and isolates´ geographic origin were not found since most isolates derived from the same localities were grouped in distinct VCGs, demonstrating their genetic variability. Further, RAPD analysis of the isolates did not reveal significant correlations between genotype, geographic location and hosts, since isolates derived from distinct localities or hosts formed the same RAPD group. Heterozygous diploids were produced as fast-growing sectors by heterokaryons formed with *M. pseudophaseolina* isolates, demonstrating, for the first time, the occurrence of the parasexual cycle in the species, based on molecular and phenotypic evidence. Diploids spontaneously produced paternal segregants and parasexual recombinants, demonstrating that parasexuality is an important mechanism for transferring genetic material in filamentous fungi. Results suggest that parasexual recombination may play an important role in the genetic variability of *M. pseudophaseolina* and may be the cause for the origin of new pathotypes, which could compromise disease control strategies in crops.

Keywords: charcoal rot, parasexual cycle, parasexual recombination, HSI phenotype, RAPD/PCR.

1 INTRODUCTION

The genus *Macrophomina* is currently recognized as a member of the family *Botryosphaeriaceae* that includes the species *Macrophomina phaseolina* (Tassi) Goid., *Macrophomina pseudophaseolina* Crous, M.P. Sarr & Ndiaye, *Macrophomina euphorbiicola* A.R. Machado, D.J. Soares & O.L. Pereira, *Macrophomina vaccinii* Zhang ter & Zhao, and *Macrophomina tecta* Vaghefi, B. Poudel & R.G. Shivas (Crous et al., 2006; Sarr et al., 2014; Machado et al., 2018; Zhao et al., 2019; Poudel et al. 2021). The species are seed- and soil-borne plant pathogens causing the charcoal rot disease in several cultivated and wild plant species including economically important crops, forest tree, fruit and weed species (Lodha & Mawar, 2020; Türkkan et al., 2020; Kaur et al., 2012; Fuhlbohm et al., 2012). Infected seeds, sclerotia, free in the soil or embedded in diseased plant tissues, are the primary source of the inoculum so that the severity of the disease is directly related to the population of viable sclerotia in the growth area (Reis et al., 2014; Kaur et al., 2012).

Charcoal rot pathogens are widely distributed in tropical and subtropical countries with semi-arid climates and are associated with damping-off, black root, and stem rot, resulting in early death of maturing plants, coupled to yield and seed quality reduction (Dhingra & Sinclair, 1978). Several management strategies, such as cultural methods, seed applied fungicides and biological control, have been studied in different parts of the world where *Macrophomina*-induced diseases are prevalent. However, such strategies have usually proved to be uneconomical or ineffective (Mengistu et al., 2015; Reis et al., 2014; Tindall, 1983). Thus, the cultivars' genetic resistance is the favoured control strategy for charcoal rot disease since it may be highly effective and may cause minimal deleterious effects on the environment (Gupta et al., 2012; Hernández-Delgado et al., 2009). Nevertheless, high genetic variability among populations of *M. phaseolina* has been reported, which may explain the difficulty of most breeding programs to find adequate levels of resistance that are reliable over the years (Reznikov et al. 2018; Romero-Luna et al. 2017; Sexton et al. 2016). In spite of *M. phaseolina*'s high degree of genetic, morphological and pathogenic variability, no sexual stage has been described in *Macrophomina* species (Iqbal and Mukhtar 2014; Sarr et al., 2014; Machado et al., 2018; Crous et al., 2006).

Genetic variations in filamentous fungi lacking a sexual stage are brought about by gene mutation, hyphal fusion, and mitotic recombination (Carlile, 1987). Genetic

recombination without sexual reproduction was first observed in *Aspergillus nidulans* in the parasexual cycle that begins with the formation of the heterokaryon. When hyphae of two genetically compatible isolates fuse together, a heterokaryotic mycelium, containing two genetically distinct nuclei, will be formed. Two distinct haploid nuclei may fuse inside the heterokaryon, resulting in the formation of a heterozygous diploid nucleus. During the divisions of diploid nucleus, mitotic recombination and mitotic nondisjunction (repeated loss of whole chromosomes) may occur to give rise to recombinant haploid nuclei containing novel allelic combinations (Strom & Bushley, 2016; Pontecorvo, 1956).

Although the parasexual cycle is indeed an effective tool to increase genotype diversity in asexual fungi, the heterokaryon's formation is a complex process that depends primarily on the ability of the pairing isolates to make hyphal anastomosis. Mutants, defective in anastomosis, named heterokaryon-self-incompatible (HSI), have been identified in several filamentous fungal species, and are characterized by their inability to undergo anastomosis or by the production of highly reduced number of hyphal fusion events (Pereira et al., 2018; Rosada et al., 2013; Glass et al., 2000). Since HSI mutants do not undergo hyphal fusions with other individuals, not even among themselves, they are unable to form heterokaryons (Glass et al., 2000). Therefore, only the wild-type isolates in anastomosis, named HSC (heterokaryon-self-compatible), will be able to form heterokaryons. Conversely, the heterokaryon viability depends on the physiological complementation between two HSC isolates, and on a specific genetic control performed by *het* (for heterokaryon incompatibility) or *vic* (for vegetative incompatibility) genes. Viable heterokaryons will be formed when HSC isolates carry identical alleles at all *het* loci, which will allocate the isolates in the same vegetative compatibility group (VCG). In contrast, if the isolates differ genetically at one or more het loci, the heterokaryotic cells will be rapidly destroyed and the isolates will be assigned in distinct VCGs (Saupe, 2000; Leslie, 1993).

In a previous study, the genetic variability of isolates of *M. phaseolina* was evaluated by vegetative compatibility groups. The ability of compatible isolates of *M. phaseolina* to form viable heterokaryons and heterozygous diploid nuclei identified the parasexual cycle in this species (Pereira et al., 2018). Since the study did not include the species *M. pseudophaseolina* and *M. euphorbiicola*, as there was no evidence of their occurrence in Brazil, and since no evidence of parasexual recombination has been reported in species of the genus *Macrophomina*, the aims of the current study are: a) to investigate the occurrence of vegetative compatibility reactions among *M.*

pseudophaseolina and *M. euphorbiicola* isolates; b) to assess the genetic variability of the Brazilian isolates of *M. pseudophaseolina* and *M. euphorbiicola* and c) to seek evidence for parasexuality in *M. pseudophaseolina* isolates by the production of heterozygous diploid nuclei and parasexual recombinants.

2 MATERIALS AND METHODS

2.1 Fungal strains and culture media

Brazilian *M. pseudophaseolina* and *M. euphorbiicola* isolates from different hosts and locations were obtained from Embrapa Algodão (Coleção de Culturas de microrganismos Fitopatogênicos, CCMF-CNPA), Universidade Federal Rural de Pernambuco, Recife PE Brazil (Coleção de Culturas de Fungos Fitopatogênicos Prof. Maria Menezes, CMM), and from Universidade Federal Rural do Semi-Árido, Mossoró RN Brazil (UFERSA) (Table 1). Media used in the current study included (Pereira et al., 2018): a) basal medium (BM; distilled H_2O, 30 g sucrose, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 10 mg $FeSO_4.7H_2O$, 15 g agar, and 0.2 mL trace element solution [95 mL distilled H_2O, 5 g citric acid, 1 g $Fe(NH_4)2(SO_4)_2.6H_2O$, 0.25 g $CuSO_4.5H_2O$, 50 mg $MnSO_4.H_2O$, 50 mg H_3BO_3, and 50 mg $Na_2MoO_4.2H_2O$]); and b) complete medium (CM; Czapeck-Dox, distilled H_2O, 10 g glucose, 2 g peptone, 0.5 g yeast extract, 1 g hydrolysed casein, 4 mg inositol, 2 mg choline chloride, 2 mg pantothenic acid, 1 mg nicotinic acid, 1 mg riboflavin, 0.1 mg 4-aminobenzoic acid, 0.5 mg folic acid, 0.5 mg pyridoxine, 0.2 mg thiamine, 2 μg biotin, and 15 g agar). Cultures were maintained at 5ºC in amber flasks.

2.2 Genomic DNA extraction

Pure cultures of *M. pseudophaseolina* and *M. euphorbiicola* were grown in liquid CM for 30 hours at 28°C. Mycelia were harvested by filtration (filter paper Whatman No. 1) and ground to a fine powder in liquid nitrogen for DNA extraction, as described by Loudon et al. (1993).

2.3 RAPD (Random Amplified Polymorphic DNA)-PCR (Polymerase Chain Reaction) amplification

PCR followed Babu et al. (2010), with some modifications, in a total volume of 25 μL containing 10 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each

of dATP, dCPT, dGTP and dTTP, 2 U of Platinum® Taq DNA polymerase (Invitrogen, Life Technologies, Brazil), 100 ng of genomic DNA and 25 ng of RAPD primer from the 16 selected 10-mer oligonucleotide primers from Operon Kit: OPA-02, OPA-08, OPA-09, OPA-10, OPA-11, OPA-12, OPA-14, OPB-07, OPB-08, OPB-17, OPV-17, OPW-02, OPW-07, OPW-08, OPW-09, and OPW-10 (Operon Technologies Inc., Alameda CA USA). Amplifications were performed with an initial denaturation step of 3 min at 94ºC; 5 cycles of 3 min at 94ºC; 3 min at 35ºC and 2 min at 72ºC, followed by 40 cycles of 1 min at 94ºC, 1 min at 36ºC, 90 sec at 72ºC, and a final extension at 72ºC for 10 min. Amplified products were analysed by electrophoresis in 2% agarose gel stained with ethidium bromide (Invitrogen, Carlsbad CA USA) and photographed under ultraviolet light on photodocumentation equipment (UVP GDS-8000 System). Only high-intensity bands reproducible in two independent amplification reactions were included in the data analyses.

2.4 Characterization of the RAPD products and data analysis

Fingerprints generated by different primers were used to analyse polymorphism between isolates and compiled by a binary system (0/1) in which (1) represents the presence of a specific band and (0) its absence. Jaccard's coefficients were clustered to generate a dendrogram using SAHN (Sequential Agglomerative Hierarchical and Nested) clustering program, selecting the unweighted pair-group method with arithmetic average (UPGMA) algorithm. The dendrogram's cut-off value was based by calculation method described by Jamshidi & Jamshidi (2011).

2.5 Isolation of mutants unable to use sodium nitrate as a nitrogen source (*nit* mutants)

So that *nit* mutants could be generated, mycelial plugs (5 mm in diameter) were removed from the edges of colonies of *M. pseudophaseolina* and *M. euphorbiicola* grown on CM and transferred to Petri plates containing BM supplemented with $NaNO_3$ (2 g/L) and $KClO_3$ (35 to 40 g/L). Plates were maintained at 28°C for 12-15 days and then visually inspected for fast-growing sectors emerging from the restricted colonies. Mycelial plugs of the fast-growing sectors were transferred to Petri plates containing BM + $NaNO_3$ and incubated at 28°C, for five days. Colonies exhibiting a thin and expanding mycelium on BM + $NaNO_3$ were classified as unable to use sodium nitrate as a nitrogen source (*nit* mutants). Mutants were submitted to phenotypic characterization in BM

supplemented with different nitrogen sources: sodium nitrate (2 g/L), potassium nitrite (0.85 g/L), hypoxanthine (0.1 g/L), uric acid (0.2 g/L) or ammonium tartrate (0.92 g/L). After a 6- to 10-day-incubation at 28°C, *nit* mutants were phenotypically analysed according to their ability to metabolize the different nitrogen sources. Mutants were then characterized as *nit1*, *nit2*, *Nit3* and *NitM*, following Brooker et al. (1991).

2.6 Heterokaryons, VCGs, auxotrophic segregants, and diploids

BM + $NaNO_3$ agar blocks (5 mm in diameter) of two genetically complementary *nit* mutants were equidistantly paired (approximately 1.5 cm) on Petri plates containing the same medium. Pairings were replicated at least twice. Plates were visually inspected for prototrophic heterokaryotic growth in the contact area of the paired mutants after 6 to 12 days of incubation at 28ºC. Isolates that showed complementation by forming heterokaryons were placed in the same VCG. On the other hand, isolates that continued to grow sparsely at the colonies' zone of contact were considered vegetatively incompatible.

Heterokaryons growing on BM + $NaNO_3$ form compact, very unstable and slow-growing colonies with irregular edges. Such heterokaryotic colonies, in turn, produced two types of segregants: (a) auxotrophic segregants exhibiting the *nit* phenotype of the paired mutants, and (b) fast-growing sectors, exhibiting prototrophic *nit* + phenotype and a growth rate similar to the original wild-type isolate. These fast-growing sectors represented diploid segregants that must be transferred to BM + benomyl (0.5 μg/ml), the haploidising agent, to test their mitotic stabilities. Diploid colonies, in contrast to heterokaryotic ones, are homogeneous, with regular edges and growth-rate similar to the wild-type isolates.

2.7 Characterization of the HSI phenotype

Genetically complementary *nit* mutants of the same isolate were paired prior to their use in inter-isolate pairings to identify HSI isolates. Hyphal fusions of supposed HSI isolates were examined with a compound light microscope (Olympus, Optical Co), following Pereira et al. (2018). Isolates were inoculated into the centre of a 90 × 15 mm Petri plate containing 12 ml 3% water agar. After 5 days of incubation at 30ºC, three 1 cm discs were cut from the colony, such that the centre of each disc was approximately 1 cm from the growing edge of the culture. Hyphal fusions were counted in each of the ten

different microscope grid fields per disc (replication) at a magnification of 200 (the total area observed for each replication was approximately 1 mm^2/disc). Resulting data for the three discs/isolate were analysed by ANOVA followed by a post hoc Bonferroni means separation ($\alpha = 0.05$) (Graphpad Prism software Version 6.0, San Diego, USA). Standard deviations of means were calculated.

3 RESULTS

3.1 Characterization of *nit* mutants and HSI phenotype

A total of 929 *nit* mutants from *M. pseudophaseolina* isolates and 177 *nit* mutants from *M. euphorbiicola* isolates were obtained in BM + $NaNO_3$ + $KClO_3$. Mutants producing poor growth colonies with little mycelial production were purified in BM + $NaNO_3$ and classified into four phenotypic classes: *nit1* (mutants for the nitrate reductase structural locus), *nit2* (mutants for the major nitrogen regulatory locus), *Nit3* (mutants for the pathway specific regulatory locus), and *NitM* (mutants defective for the molybdenum cofactor loci) (Brooker et al., 1991). Among the *M. pseudophaseolina* isolates, *nit1* was the predominant phenotypic class for most (44.5 %) isolates, followed by *NitM* (29.6%), *Nit3* (22.2%), and *nit2* (3.7%). Whilst the most frequent mutants obtained for isolate 28 were also *nit1* in the case of *M. euphorbiicola*, they were *Nit3* for isolates 29 and 30. At least two different classes of *nit* mutants were obtained for each wild-type isolate (Table 2).

Subsequently, the genetically complementary *nit* mutants from the same parent isolate were paired to identify the occurrence of the HSI phenotype among isolates of *M. pseudophaseolina* and *M. euphorbiicola*. Since isolates 2, 5 (*M. pseudophaseolina*), and 28 (*M. euphorbiicola*) failed to show any intra-isolate complementation, they were classified as HSI isolates. Light microscope examination of hyphal fusions of isolates 12 (HSC), 2 (HSI), and 5 (HSI) from *M. pseudophaseolina*, and isolates 30 (HSC) and 28 (HSI) from *M. euphorbiicola* revealed that the average number of hyphal fusions/mm^2 for isolates 2 ($0.17 \pm 235\ 0.15$) and 5 (0.08 ± 0.14) were significantly ($p < 0.0001$) lower than that observed for isolate 12 (8.9 ± 0.39). Likewise, the average number of hyphal fusions/mm^2 for isolate 28 (0.1 ± 0.1) was significantly ($p < 0.0001$) lower when compared with that from isolate 30 (4.5 ± 0.6) (Fig. 1c). Based on these results, isolates 2, 5, and 28 were characterized as HSI and, consequently, they could not be classified in any of the identified vegetative complementation groups (Fig. 1, Table 2).

3.2 Characterization of the *Macrophomina* isolates in VCGs

No heterokaryotic mycelia were observed when complementary *nit* mutants of *M. euphorbiicola* isolates 29 and 30 were paired in BM + $NaNO_3$ plates. Further, isolates 29 and 30 did not form heterokaryons with any of the HSC isolates of *M. pseudophaseolina* (Table 2, Fig. 2a).

By pairing complementary *nit* mutants of the HSC isolates of *M. pseudophaseolina* in all possible combinations in BM + $NaNO_3$, 18 distinct VCGs were identified, specifically 13 VCGs consisted of a single isolate (VCGs A, B, E, F, I-P, and R), three consisted of two isolates (VCGs C, G, and Q), one consisted of three isolates (VCG H) and one (VCG D) contained four isolates (Table 2).

Most of the *M. pseudophaseolina* isolates were allocated in distinct VCGs, although they were mostly obtained from a single growing area of the same municipality. Exceptions were related to VCGs C and Q only, represented by at least two isolates from the same locality. On the other hand, VCGs D, G, and H included isolates obtained from distinct localities, albeit from the same host (G and H) and even from distinct localities and distinct hosts (D). Curiously, VCG D included isolates obtained from 3 distinct localities (Tables 1 and 2).

All HSC isolates from *M. pseudophaseolina* formed 259 strong heterokaryotic reactions in intra-isolate and inter-isolate pairings, except isolate 4 which produced strong heterokaryons with *nit* mutants from isolates 12, 13, and 18 from VCG-D, and weak, very slow and frequently discontinuous heterokaryons with *nit* mutants from isolate 6 (VCG-C). This suggests that isolate 4 forms a bridge between VCGs C and D (Fig 2b, c).

If the pairing of *nit* complementary mutants was separated by a sterilized dialysis membrane, no heterokaryon was formed, indicating the need for physical contact between complementary mutants for the heterokaryon formation (Fig. 2d). In addition, when mycelial plugs from heterokaryons were removed and transferred to BM + $NaNO_3$, *nit* segregants, exhibiting the auxotrophies of the paired mutants, were produced (Table 3, Fig. 2e). The above demonstrated that the prototrophic growth of the heterokaryons is not a result of reversion to prototrophy, but due to heterokaryosis.

3.3 Diploid formation

The heterokaryon formed by pairing of complementary *nit* mutants from isolate 14 and by complementary *nit* mutants from isolates 4 and 6 produced separately two fast

growing sectors, with prototrophic (*nit* +) phenotypes when growing in BM + $NaNO_3$. Both sectors were isolated, purified, and transferred to BM + $NaNO_3$ where they formed prototrophic and homogeneous colonies, with regular edges and growth rates similar to the parent wild-type isolates (Fig. 2f). When tested for their mitotic stability in BM + $NaNO_3$ + benomyl, both prototrophic sectors produced mitotic segregants with the *nit* phenotypes of the original paired mutants. Prototrophic sectors were characterized as heterozygous diploids and named respectively D14//14 and D4//6. When growing in BM + $NaNO_3$, diploids D14//14 and D4//6 produced spontaneously recombinant prototrophic segregants, named R1 and R2, respectively, both with *nit*+ phenotype (Table 3, Fig. 2f). The haploid condition of the prototrophic recombinant was demonstrated by its mitotic stability when growing in BM + $NaNO_3$ + benomyl, without the production of new mitotic sectors.

3.4 Molecular characterization of heterokaryon, diploid, and prototrophic recombinant

Genetic polymorfism could be identified between the wild isolate 14, diploid D14//14, heterokaryon 14-*nit1*/14-*Nit3* from isolate 14, and R1 prototrophic recombinant. In the RAPD analysis obtained with primer OPW-07 (5´-CTGGACGTCA-3´) a band of approximately 900 bp was amplified from wild isolate 14, heterokaryon 14-*nit1*/14-*Nit3*, and recombinant R1, but it was not identified in diploid D14//14 (Fig. 3a). Further, bands of approximately 250 bp and 700 bp were amplified only from heterokaryon 14-*nit1*/14-*Nit3* (Figure 3a). In the RAPD profile performed with primer OPW-09 (5´-GTGACCGAGT-3´), polymorphic bands, with approximately 650 bp and 800 bp, were amplified from isolate 14 and heterokaryon 14-*nit1*/14-*Nit3*, but not from diploid D14//14 and R1 prototrophic recombinant (Figure 3b). Results demonstrated that RAPD analysis was useful to distinguish genomic differences among *M. pseudophaseolina* diploid, heterokaryotic, and prototrophic recombinant colonies.

3.5 Molecular characterization of *Macrophomina* isolates

The RAPD analysis was based on polymorphic bands obtained with nine (OPA-02, OPA-09, OPA-10, OPB-08, OPB-17, OPW-02, OPW-07, OPW-08 and OPW-10) of the 16 selected random primers (Supplementary Fig. S1, Supplementary Table S1). The UPGMA clustering produced a dendrogram that separated the *M. pseudophaseolina* and *M. euphorbiicola* isolates into four RAPD groups (I to IV), and the dendrogram's cut-off

value was calculated as 65% of genetic similarity (Fig. 4). The genetic similarity rate among the isolates ranged between 50.3% and 92.5%. Group I was formed by 26 out of the 27 *M. pseudophaseolina* isolates, with 69.6% similarity. In this group, two subgroups had been identified: Ia was composed of isolates 1, 2, 5-15, 17, 18, 20-24, 26, 27, and Ib was composed of isolates 3, 4, 16, 19, showing similarity coefficients of 75.4% and 74.2%, respectively. The two subgroups comprised isolates from different hosts and geographic areas. Group II was composed of *M. pseudophaseolina* isolate 25 only, with genetic similarity of 58% in relation to Group I (Fig. 4). *M. euphorbiicola* isolates were allocated in Groups III and IV. Group III was formed by isolates 28 and 29, both derived from different hosts and localities, with 70.2% of genetic similarity. On the other hand, Group IV was formed only by isolate 30, sharing a 54.6% genetic similarity in relation to Group III. The genetic similarity between species *M. pseudophaseolina* (Groups I and II) and *M. euphorbiicola* (Groups III and IV) was 50.3% (Fig. 4).

4 DISCUSSION

The current study demonstrates the occurrence of vegetative compatibility reactions and HIS phenotypes in *M. pseudophaseolina* and *M. euphorbiicola*. For the first time, it has been shown that the parasexual cycle does occur in *M. pseudophaseolina*, with the production of mitotic recombinants.

Mutants HSI have been described in several phytopathogenic fungi, including *Giberella fujikuroi*, *Aspergillus flavus,* and *M. phaseolina* (Correl et al., 1989; Rosada et al., 2013; Pereira et al., 2018), and, in the current research work, in *M. pseudophaseolina* (isolates 2 and 5) and *M. euphorbiicola* (isolate 28). Although HSI mutants may eventually establish anastomosis with HSC isolates, such mutants cannot be classified into groups of vegetative compatibility (Pereira et al., 2018). In spite of this fact, the HSI mutants 2, 5, and 28 showed several RAPD markers in common with isolates from VCGs A, C-J, H, L-P, Q, R (isolates 2 and 5), and S (isolate 28).

The hyphal anastomosis process has been described as a highly unstable and strain dependent characteristic. Statistically significant variations in the frequency of hyphal anastomosis among HSC isolates, have been previously described in *A. flavus* and *Fusarium solani* (Rosada et al., 2013; Hawthorne & Rees-George, 1996). In the current study, statistically significant variations in the number of hyphal anastomosis of HSC isolates from *M. pseudophaseolina* and *M. euphorbiicola* (isolates 12 and 30,

respectively) were reported. On the other hand, hyphal anastomosis of HSI mutants was reported to be often less frequent than their counterpart HSC in *M. euphorbiicola* and *M. pseudophaseolina*.

The pairing of genetically complementary *nit* mutants of 25 *M. pseudophaseolina* HSC isolates identified 18 VCGs, most of which (72%) were characterized as single member VCGs. Isolates belonging to the same VCG normally have identical alleles at their compatibility loci (*het*), while the occurrence of gene mutations in these loci make the isolates vegetatively incompatible and allocate them in different VCGs (Leslie, 1993). It is conceivable that *M. pseudophaseolina* isolates that now belong to single-member VCGs may have been members of a major VCG. However, when undergoing a simple mutation at one or more loci *het*, they could no longer form heterokaryons with the other members (Elias & Schneider, 1991). VCG diversity, or rather, the number of VCGs/number of isolates (Caten & Newton, 2000), of *M. pseudophaseolina* isolates was 0.72. A high VCG diversity (0.83) has been reported in previous research with *M. phaseolina*. It has been hypothesized that such results were associated with the paradoxical dispersal capacity of this fungus (Pereira et al., 2018), where, albeit with limited dispersal ability within a single area, it acts as a monocyclic pathogen. At the same time, it may be dispersed to long distances by infected seeds. Results herein obtained with *M. pseudophaseolina* and *M. euphorbiicola* strengthen this hypothesis and explain why the vast majority of isolates evaluated were allocated in distinct VCGs, even though exceptionally some isolates from distinct hosts and location were grouped in a single VCG.

Correlations between VCG and geographic origin of the isolates from *M. euphorbiicola* and *M. pseudophaseolina* were not found since most isolates derived from the same localities were grouped in distinct VCGs. In *M. pseudophaseolina*, only three multimember VCGs (VCG-D, VCG-G, and VCG-H) were found to group isolates belonging to different localities. Similarly, RAPD analysis did not reveal significant correlations between genotype, geographic location, and hosts since *M. pseudophaseolina* isolates from distinct localities (10-15, 17, 18, 20-24, 26, and 27) formed the RAPD subgroup Ia. In contrast, isolates 16 and 25, obtained from two different growing areas from the same municipality, formed the RAPD Groups Ib and II, respectively, suggesting the occurrence of genetic variability among the isolates.

Isolates 22 and 23, from the same geographic area, and isolates 5 and 17, from distinct localities, exhibited the highest similarity coefficients (92.5% and 91.8%,

respectively, subgroup Ia). It is also important to highlight that the vegetative compatibility of the isolates did not show any correlation with RAPD analysis. *M. pseudophaseolina* isolates 17 and 18, respectively from VCGs H and D, were allocated in RAPD subgroup Ia, with 89.4% similarity. On the other hand, isolates 25 and 26, from VCG-Q and derived from the same host and locality, were allocated in different RAPD Groups (II and Ia, respectively), with 63.8% similarity. Additionally, current analysis demonstrated that *M. pseudophaseolina* isolates from RAPD subgroup Ia (22 isolates) were distributed into 15 distinct VCGs and thus demonstrated the ability of VCG analysis to differentiate genotypically similar isolates.

In the current study, isolate 4 was interestingly able to form heterokaryons with isolates from VCG-D and VCG-C, and was characterized as a "bridging" isolate. Bridging isolates, previously described in *Colletotrichum lindemutianum*, *Fusarium oxysporum* f. sp. *radicislycopersici,* and *Verticillium dahlia*, may exchange genetic information between different VCGs, since the complete genetic isolation among these VCGs has not yet developed (Rodrigues-Guerra et al., 2003; Ebihara et al., 1999; Katan et al., 1991). Our results suggest some degree of genetic relatedness between isolates from VCGs C and D to allow for the formation of viable heterokaryons between isolates from the two VCGs.

Diploid nuclei formed with isolates 4 and 6 (D4//6) and with mutants *nit1* and *Nit3* from isolate 14 (D14//14) were noticeably obtained from the heterokaryons. Diploids (D4//6 and D14//14) resulting from the fusion of paternal haploid nuclei were heterozygous and showed mitotic instability, giving rise to segregants with both paternal (*nit*) and recombinant (*nit*+) phenotypes. In the parasexual cycle, diploid nuclei may undergo mitotic crossing-over and chromosomal losses (haploidization), originating haploid recombinants. If, however, mitotic crossing-over fails to occur, haploid recombinants may still be produced, during the haploidization of the diploid nucleus, through the independent assortment of the paternal chromosomes (Strom & Bushley, 2016; Souza-Júnior et al., 2007). In *Alternaria solani*, recombinant haplotypes were obtained in laboratory conditions by the haploidization of heterozygous diploid nuclei, suggesting that parasexuality is likely to occur in this species (Zhao et al., 2021). An atypical parasexual cycle, named parameiosis, has been described in some filamentous fungi that produces haploid recombinants directly from heterokaryons. The process comprises the formation of very unstable diploid nuclei inside the heterokaryotic mycelium, where they undergo mitotic recombination and haploidization. In contrast to

the parasexual cycle, the diploid phase cannot be recovered in parameiosis (Sybuia et al., 2020; Paccola Meirelles & Azevedo, 1991). Parasexuality is therefore a widespread mechanism for transferring genetic material in filamentous fungi which may contribute towards the survival of the fungus species under adverse conditions through the production of new genotypes (Sybuia et al., 2020; Souza-Paccola et al., 2003). Consequently, the genetic diversity resulting from parasexual recombination in phytopathogens may be responsible for the origin of new pathotypes, compromising the strategies for disease control in crops (Noguchi et al., 2006).

The current study demonstrates not only the occurrence of vegetative compatibility reactions in *M. pseudophaseolina* and *M. euphorbiicola* isolates but also the ability of *M. pseudophaseolina* isolates to produce heterozygous diploid nuclei and parasexual recombinants. The characterization of the parasexual cycle in *M. euphorbiicola* was not possible due to the small number of isolates analyzes (totaling 3 only). Further, one of the *M. euphorbiicola* isolates was characterized as HSI (isolate 28), and two of them (29 and 30), although phenotypically characterized as HSC, did not show vegetative compatibility. Thus, further studies involving a larger number of *M. euphorbiicola* HSC isolates are needed to characterize the parasexuality in this species.

Since the most effective charcoal rot management strategies are based on the development of resistant cultivars (Iqbal & Mukhtar, 2014; Gupta et al., 2012), the exchange of genetic information between vegetatively compatible isolates through parasexual recombination may be responsible for the breakdown of such resistance, restoring the pathogen´s aggressiveness or infectivity. Therefore, results suggest that the parasexual recombination in *M. pseudophaseolina* may play an important role in the genetic variability of the species.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1. Host, geographical origin, identity, source and GenBank accession numbers of *M. pseudophaseolina* and *M. euphorbiicola* isolates used in the current study

Isolates	Host/Species	Geographic location (Municipality/State) [a]	Confirmed Molecular Identity	Source		GenBank Accession Numbers (Tef1-alpha)
				Culture Collections	Original Code	
1	*Ricinus communis*	Central/BA	*M. pseudophaseolina*	Embrapa algodão	CCMF-CNPA 291	KU058909
2	*Gossypium hirsutum*	Apodí/RN	*M. pseudophaseolina*	Embrapa algodão	CCMF-CNPA 293	KU058911
3	*G. hirsutum*	Apodí/RN	*M. pseudophaseolina*	Embrapa algodão	CCMF-CNPA 294	KU058912
4	*Arachis hypogaea*	Apodí/RN	*M. pseudophaseolina*	Embrapa algodão	CCMF-CNPA 667	KU058919
5	*A. hypogaea*	Apodí/RN	*M. pseudophaseolina*	Embrapa algodão	CCMF-CNPA 668	KU058920
6	*A. hypogaea*	Apodí/RN	*M. pseudophaseolina*	Embrapa algodão	CCMF-CNPA 669	KU058921
7	*Trianthema portulacastrum*	Icapuí/CE	*M. pseudophaseolina*	UFERSA	CMM 4766	MH373507
8	*T. portulacastrum*	Icapuí/CE	*M. pseudophaseolina*	UFERSA	CMM 4767	MH373513
9	*T. portulacastrum*	Icapuí/CE	*M. pseudophaseolina*	UFERSA	CMM 4768	MH373468
10	*T. portulacastrum*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4770	MH373470
11	*T. portulacastrum*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4771	MH373471
12	*T. portulacastrum*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4772	MH373514
13	*T. portulacastrum*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4773	MH373472
14	*T. portulacastrum*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4774	MH373512
15	*T. portulacastrum*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4775	MH373473

Table 1. Continued

16	*T. portulacastrum*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4778	MH373509
17	*T. portulacastrum*	Mossoró/RN	*M. pseudophaseolina*	UFERSA	CMM 4781	MH373476
18	*T. portulacastrum*	Mossoró/RN	*M. pseudophaseolina*	UFERSA	CMM 4782	MH373478
19	*T. portulacastrum*	Mossoró/RN	*M. pseudophaseolina*	UFERSA	CMM 4783	MH373477
20	*T. portulacastrum*	Mossoró/RN	*M. pseudophaseolina*	UFERSA	CMM 4784	MH373479
21	*T. portulacastrum*	Mossoró/RN	*M. pseudophaseolina*	UFERSA	CMM 4787	MH373482
22	*Boerhavia difusa*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4796	MH373491
23	*B. difusa*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4797	MH373492
24	*B. difusa*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4798	MH373493
25	*B. difusa*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4799	MH373494
26	*B. difusa*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4800	MH373516
27	*B. difusa*	Assú/RN	*M. pseudophaseolina*	UFERSA	CMM 4801	MH373517
28	*Jatropha gossypifolia*	Lagoa Seca/PB	*M. euphorbiicola*	Embrapa algodão	CCMF-CNPA 278	KU058898
29	*R. communis*	Irecê/BA	*M. euphorbiicola*	Embrapa algodão	CCMF-CNPA 288	KU058906
30	*R. communis*	Irecê/BA	*M. euphorbiicola*	Embrapa algodão	CCMF-CNPA 289	KU058907

BA, RN, CE, PB = Bahia, Rio Grande do Norte, Ceará and Paraíba States, Brazil. UFERSA= Universidade Federal Rural do Semi-Árido, Mossoró-RN, Brazil. Isolates 1-6 and 28-30 were provided by Dr. Dartanhã J. Soares. Isolates 9-27 were kindly donated by Dr. Rui S. Júnior.

Table 2. Number (N_o) and frequencies (%) of *nit* mutants (*nit1*, *nit2*, *Nit3* and *NitM*), vegetative compatibility groups (VCG), and HSC or HSI phenotype of *M. pseudophaseolina* and *M. euphorbiicola* isolates.

Isolates	*nit1*		*nit2*		*Nit3*		*NitM*		VCGs	HSC/HSI
	No	%	No	%	No	%	No	%		
1	13	39.4	0	0	20	60.6	0	0	A	HSC
2	50	82	0	0	7	11.5	4	6.5	nd	*HSI*
3	28	57.2	0	0	15	30.6	6	12.2	B	HSC
4	10	55.6	0	0	8	44.4	0	0	C/D	HSC
5	24	34.8	0	0	3	4.3	42	60.9	nd	*HSI*
6	0	0	14	42.4	19	57.6	0	0	C	HSC
7	11	44	3	12	11	44	0	0	E	HSC
8	9	39.1	1	4.4	12	52.1	1	4.4	F	HSC
9	6	20	4	13.3	19	63.4	1	3.3	G	HSC
10	3	13	1	4.4	19	82.6	0	0	H	HSC
11	2	7.4	13	48.1	12	44.5	0	0	G	HSC
12	7	31.8	1	4.5	6	27.3	8	36.4	D	HSC
13	9	56.2	0	0	3	18.8	4	25.0	D	HSC
14	16	61.6	0	0	5	19.2	5	19.2	I	HSC
15	10	50	0	0	10	50	0	0	J	HSC

Table 2. Continued.

16	32	84.2	0	0	0	0	6	15.8	H	HSC
7	0	0	6	23.1	9	34.6	11	42.3	H	HSC
18	2	3.7	13	24.1	4	7.4	35	64.8	D	HSC
19	11	32.3	0	0	9	26.4	14	41.1	K	HSC
20	12	24	9	18	10	20.0	19	38.0	L	HSC
21	12	33.3	0	0	2	5.6	22	61.1	M	HSC
22	13	56.5	0	0	10	43.5	0	0	N	HSC
23	15	45.4	5	15.2	4	12.1	9	27.3	O	HSC
24	6	24	0	0	2	8	17	68	P	HSC
25	14	34.2	3	7.3	13	31.7	11	26.8	Q	HSC
26	42	79.2	0	0	1	1.9	10	18.9	Q	HSC
27	30	73.2	8	19.5	3	7.3	0	0	R	HSC
28	23	33.8	3	4.4	21	30.9	21	30.9	nd	*HSI*
29	8	19	1	2.4	31	73.8	2	4.8	S	HSC
30	0	0	5	7.5	39	58.1	23	34.4	T	HSC

Nd= Not defined

Table 3. Number and phenotypes of mitotic segregants derived from heterokaryon formed with isolates 16 and 17, and from diploids D14//14 and D4//6.

Heterokaryon	Pairing of *nit* mutants		Number and phenotypes of mitotic segregants derived from heterokaryons		
	Paternal 1 // Paternal 2		Paternal 1	Paternal 2	Total
16 / 17 [a]	Isolate 16 (*nit1*) // Isolate 17 (*NitM*)		15 (*nit1*)	21 (*NitM*)	36
Diploids	Pairing of *nit* mutants		Number and phenotypes of mitotic segregants derived from diploids		
	Paternal 1 // Paternal 2		Paternal 1	Paternal 2	Recombinants (*nit*$^{+}$)	Total
D14//14 [b]	Isolate 14 (*Nit3*) // Isolate 14 (*nit1*)		23 (*Nit3*)	6 (*nit1*)	01	29
D4//6 [a]	Isolate 4 (*nit1*) // Isolate 6 (*Nit3*)		25 (*nit1*)	19 (*Nit3*)	01	44

[a] Inter-isolate pairing; [b] Intra-isolate pairing

Table S1. RAPD banding pattern of *M. pseudophaseolina* and *M. euphorbiicola* isolates used in this study.

	Band Sizes Range (bp)								
	Primers								
Isolates	OPA-02	OPA-09	OPA-10	OPB-08	OPB-17	OPW-02	OPW-07	OPW-08	OPW-10
1	550-2100	200-2000	700-1600	350-2100	300-1300	800-900	390-1600	600-2800	1000
2	550-2100	200-2000	850-1800	350-2100	290-1480	800-1700	390-2000	600-2800	1000-1400
3	520-1500	200-2000	650-1400	350-2100	650	800-1700	390-1600	600-2200	1000
4	850- 1400	200-2000	650-1400	350-2100	300-1300	800-900	410-1600	600-1100	1000
5	550-2100	200-2000	700-1600	350-2100	300-1300	800-1700	390-1600	600-2800	1000-1400
6	550-2100	200-2000	650-1300	350-2100	300-1300	800-1700	410-1600	600-2200	1000
7	550-2100	200-2000	700-1600	350-2100	300-1300	800-1700	390-1600	600-2200	1000
8	550-2100	200-2000	850-1400	350-2100	300-1480	800-1700	390-1600	600-2800	1000-1400
9	550-2100	200-2000	850-1600	350-2100	300-1300	800-1700	390-1100	600-2800	1000
10	550-2100	200-2000	900-1600	350-1200	300-1300	800-1700	390-1600	600-2200	1000-1400
11	550-2100	200-2000	700-1600	350-2100	300-1300	800-1700	390-1600	600-2800	1000-1400
12	520-1500	200-2000	650-1600	350-1200	300-1000	800-1700	390-1600	600-2800	1000
13	550-1400	200-2000	650-1600	350-2100	650	800-1700	390-1600	600-2800	1000
14	550-1400	200-2000	650-1600	350-2100	300-650	800-1700	390-1600	600-2200	1000
15	550-1800	200-2000	450-2100	350-2100	300-1480	800-1700	390-1600	600-2800	1000
16	520-1400	200-2000	650-1600	350-1200	300-1300	800-1700	390-1600	600-2800	1000
17	550-1400	200-2000	700-1400	350-2100	300-1300	800-1700	390-1600	600-2800	1000-1400
18	550-1800	200-2000	700-1700	350-2100	300-1300	800-1700	390-1600	500-2800	1000-1400
19	520-850	200-2000	650-1400	350-1200	300-1300	800-1700	390-1600	600-950	1000
20	520-1400	200-2000	650-1500	350-2100	300-1480	800-1700	390-1600	600-2800	1000-1400
21	520-1500	200-2000	700-1500	350-2100	290-1480	800-1700	390-1600	600-2800	1000-1400
22	520-1800	200-2000	700-1700	350-2100	300-1480	800-1700	390-1600	500-2800	1000-1400
23	520-2900	200-2000	700-1500	350-2100	300-1480	800-1700	390-1600	600-2800	1000-1400
24	520-1500	200-2000	650-1500	350-2100	650-1480	800-1100	390-1600	500-2800	1000-1400
25	420-1800	200-2000	700-1900	350-2100	300-1480	490-1300	390-2000	500-2800	600-1400
26	450-1400	200-2000	700-1700	350-2100	300-1300	800-1700	390-1600	500-2800	1000-1400
27	450-1800	200-2000	7001700	350-2100	300-1300	800-1700	390-1600	500-2800	1000-1400
28	850-1100	200-2000	600-1700	350-2100	490-1600	490-1700	390-2000	500-2800	500-1000
29	640-1400	200-2000	600-1700	350-2100	490-1600	600-1700	390-2000	600-2800	500-1600
30	640-1500	200-2000	650-1700	350-750	490-1480	490-1500	390-1200	600-950	500-1000
NPB[a]	11	6	16	9	13	10	11	13	6
NMB[b]	0	11	1	1	0	0	4	2	1
PPL (%) [C]	100	35	94	90	100	100	73	86	86

NPB[a] = Number of polymorphic bands; NMB[b] = Number of monomorphic bands; PPL[c]= proportion of polymorphic loci.

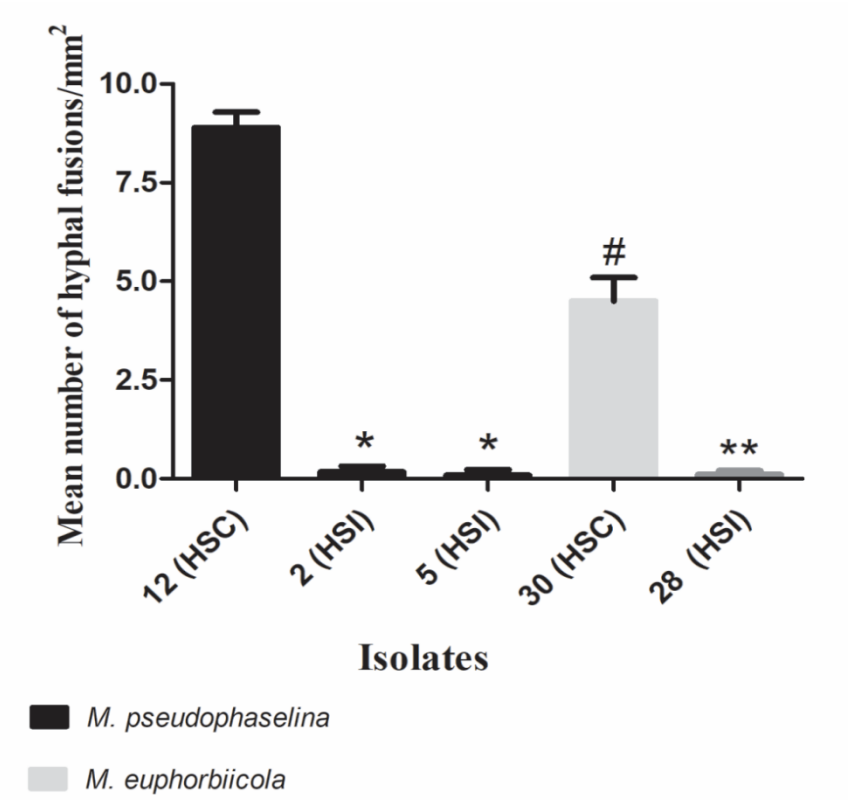

Fig. 1 Mean number of hyphal fusions/mm2 of the HSC isolates 12 (*M. pseudophaseolina*) and 30 (*M. euphorbiicola*), and of the HSI isolates 2, 5 (*M. pseudophaseolina*) and 28 (*M. euphorbiicola*). Column heights indicate means ± standard deviations of three experiments. (*) denotes statistical differences between HSI isolates 2 and 5 and HSC isolate 12. (**) denotes significant difference between HSI isolate 28 and HSC isolate 30. (#) denotes significant difference between HSC isolates 30 and 12. Data were analysed by ANOVA followed by a post hoc Bonferroni means separation ($p < 0.0001$**).**

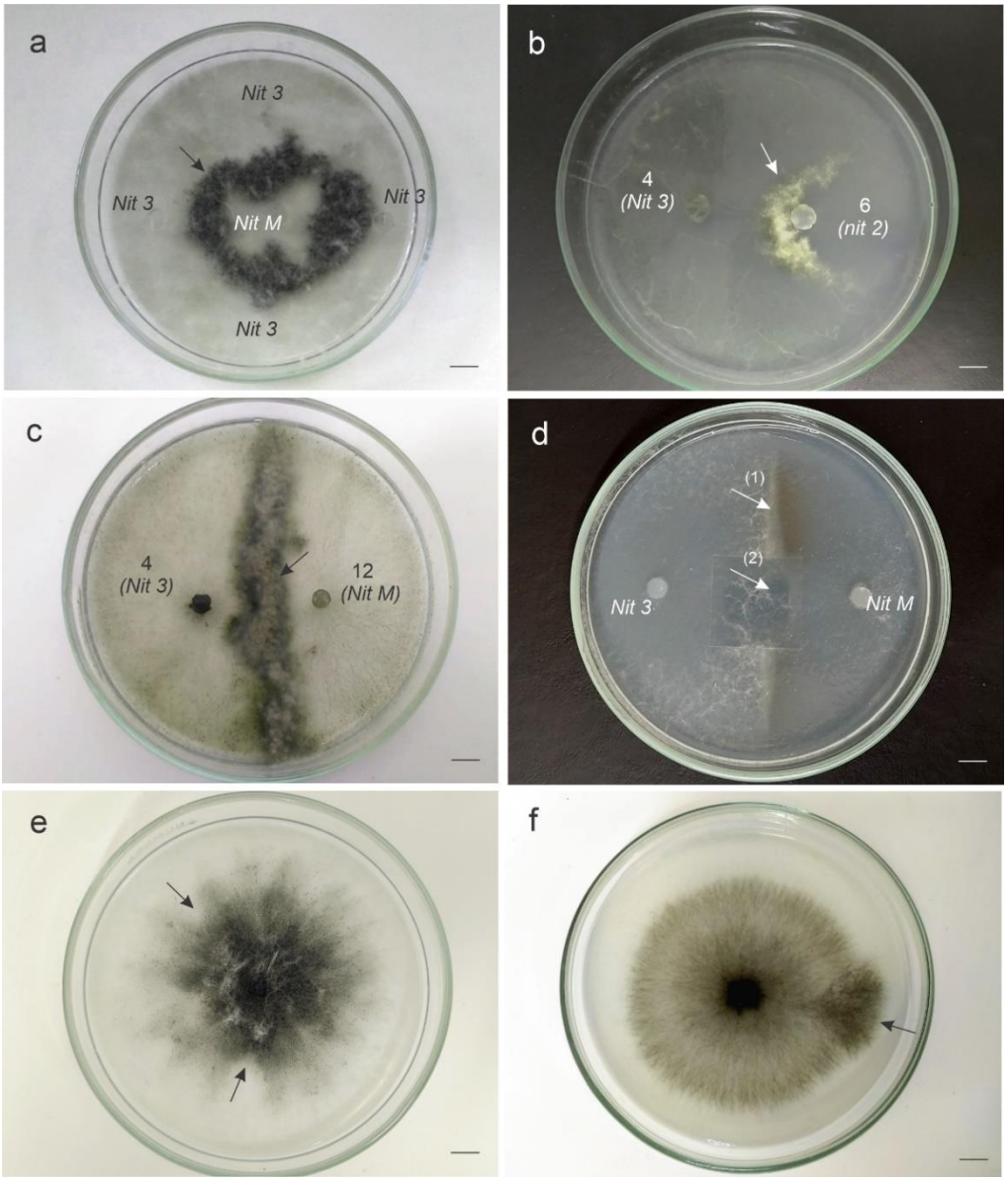

Fig. 2 Heterokaryotic and diploid colonies formed between complementary *nit* mutants of *M. euphorbiicola* and *M. pseudophaseolina* isolates. (a) Heterokaryon (arrow) formed by complementary *nit* mutants from isolate 30 of *M. euphorbiicola*; (b) Heterokaryon (arrow) formed by isolates 4 and 6 (VCG C) in BM + $NaNO_3$ after 16 days of incubation at 28ºC; (c) Heterokaryon (arrow) formed by isolates 4 and 12 (VCG D) in BM + $NaNO_3$ after 8 days of incubation at 28ºC; (d) Heterokaryon (1) formed between *Nit3* and *NitM* mutants from *M. pseudophaseolina* isolate 18. Heterokaryon was not formed (2) when mutants *Nit3* and *NitM* were separated by a sterilized dialysis membrane which impaired direct contact between the pairing mycelia; (e) Heterokaryotic colony, formed by the pairing of complementary *nit* mutants from isolates 4 and 6 of *M. pseudophaseolina*, growing in BM + $NaNO_3$ after 4 days of incubation at 28ºC. Colony shows mitotic instability (arrows) and irregular edges; (f) Diploid colony (D4//6) growing in BM + $NaNO_3$ after 3 days of incubation at 28ºC. Colony produced a parasexual recombinant *nit*+ (arrow), revealing mitotic stability and regular edges. Bar = 5.0 mm.

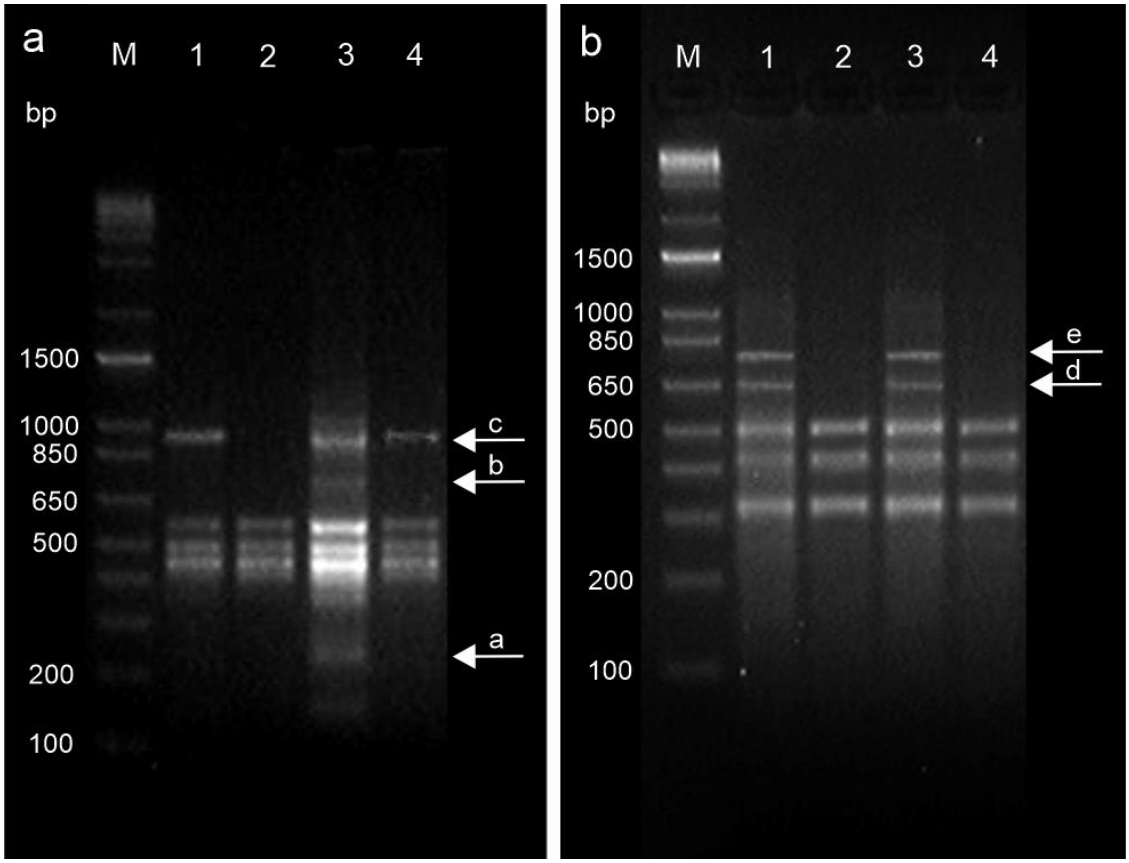

Fig. 3 RAPD profiles of isolate 14, diploid D14/14, heterokaryon *nit1/Nit3*, and recombinant R1 (lines 1, 2, 3, 4) in agarose gel using primers OPW-07 (a) and OPW-09 (b). Column M = molecular weight DNA ladder in bp (100 – 1500), bp = base pairs. Arrows indicate polymorphic bands of 250 bp (a), 700 bp (b), 900 bp (c), 650 bp (d), and 800 bp (e).

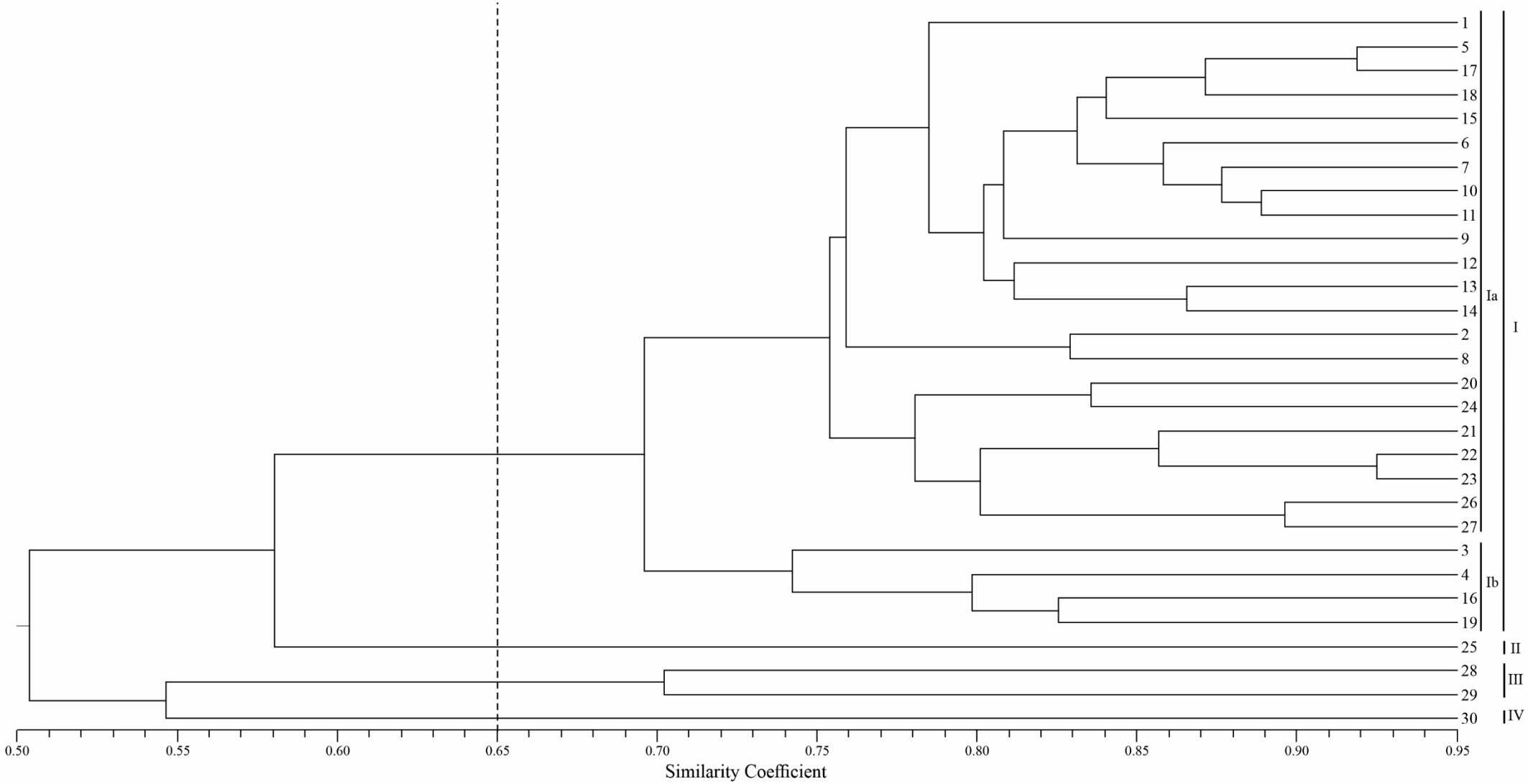

Fig. 4 Dendrogram (NTSYS software) constructed with UPGMA clustering method from *M. pseudophaseolina* (totaling 27 isolates) and *M. euphorbiicola* isolates (totaling 3 isolates), using 9 different primers and RAPD analysis). Similarities were computed from 136 random polymorphic bands. I to IV = RAPD groups; Ia and Ib = RAPD subgroups. The scale in the figure is the genetic similarity coefficient calculated according to Jaccard.

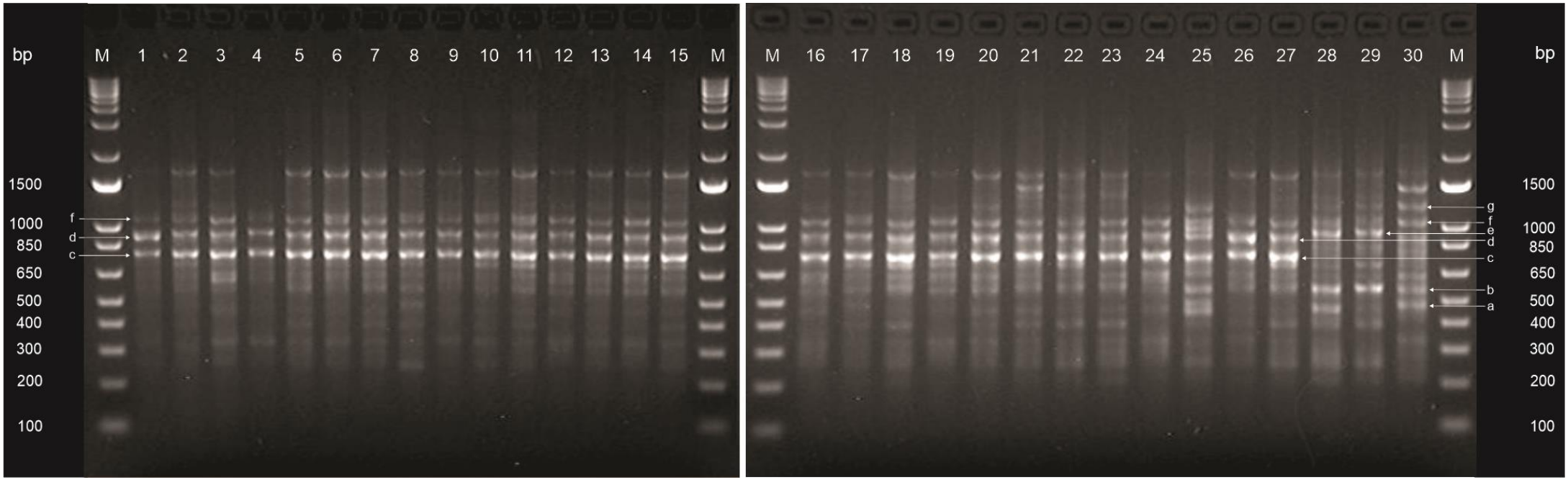

Fig. S1 RAPD products of *M. pseudophaseolina* (1-27) and *M. euphorbiicola* (28-30) isolates using the primer OPW-02. Column M = molecular weight DNA ladder in bp (100 –1500), bp = base pairs. Arrows indicate polymorphic bands of 490 bp (a), 600 bp (b), 800 bp (c), 900 bp (d), 950 bp (e), 1,100 bp (f), and 1,300 bp (g).