Heterokaryosis and diploid formation among Brazilian isolates of *Macrophomina phaseolina*

Tais Susane Pereira[a], Giovana Natielle Machado Esquissato[a], Claudinéia Conationi da Silva Franco[a], Paulo Cezar de Freitas Mathias[a], Dartanhã José Soares[b], Clive Howard Bock[c], William Mário de Carvalho Nunes[d], Carlos Alexandre Zanutto[d], and Marialba Avezum Alves de Castro-Prado[a*]

[a]Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Avenida Colombo 5790. Maringá, PR 87020-900, Brazil; [b]Empresa Brasileira de Pesquisa Agropecuária (Embrapa Algodão), Rua Osvaldo Cruz, n° 1.143. Campina Grande, PB 58428-095, Brazil; [c]USDA-ARS-SEFTNRL, Byron, Georgia, USA; [d]Departamento de Agronomia, Universidade Estadual de Maringá, Avenida Colombo 5790. Maringá, PR 87020-900, Brazil.

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Running head: Parasexuality in *Macrophomina phaseolina*

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[*]Corresponding author: M.A.A. de Castro-Prado; E-mail address: maacprado@gmail.com

ABSTRACT

Heterokaryosis is the association of genetically distinct nuclei in a common hyphal cytoplasm, and is a process involved in the generation of fungal variation. The fusion of the distinct nuclei within a heterokaryotic hypha produces a heterozygous diploid nucleus and is one of the stages of the parasexual cycle. A heterokaryon's viability depends on the isolates' genetic constitution with regard to loci called *het* (heterokaryon incompatibility) or *vic* (vegetative incompatibility). The current study evaluated for the first time the diversity of vegetative compatibility reactions in isolates of *Macrophomina phaseolina* from different hosts. Complementary *nit* (nitrate non-utilizing) mutants of each isolate were obtained and paired in all possible combinations. Isolates were classified in vegetative compatibility groups (VCG) according to their ability to form viable heterokaryons. Ten VCGs were identified, two of them contained 2 isolates, and the remainder contained a single isolate. When growing on basal medium (BM), heterokaryons produced both: (*a*) auxotrophic segregants exhibiting the same phenotype of the paired mutants and (*b*) a fast-growing sector, characterized as a heterozygous diploid sector, named D653, which showed a *nit+* phenotype with a growth rate similar to the original wild isolate. When growing in the presence of benomyl, the haploidizing agent, D653 produced auxotrophic haploid segregants exhibiting the *nit* phenotypes of the crossed mutants. The results demonstrate for the first time the ability of *M. phaseolina* isolates to form viable heterokaryons and heterozygous diploid nuclei, suggesting that the parasexual cycle may be an alternative source of the genetic variability in this species.

Introduction

Macrophomina phaseolina (Tassi) Goid. is the causal agent of charcoal rot of several important crops, including soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), cotton (*Gossypium hirsutum*) and corn (*Zea mays*) (Mayek-Pérez *et al*., 2002; Almeida *et al*., 2003; Gupta *et al*., 2012; Coser *et al*., 2016; Ramos *et al*., 2016). *M. phaseolina* is seed- and soil-borne and produces black

microsclerotia in the root and stem tissue of its host, enabling it to survive in adverse conditions. The microsclerotia in soil, infected seeds or host tissues serve as primary inoculum (Reis *et al*., 2014). The most effective method to control the disease is the use of resistant cultivars, and emphasis has been placed on development of new resistant germplasm. Although no charcoal rot-resistant variety of soybeans is available, a resistant common bean germplasm has been described, (BAT 477), that has two dominant genes with double-recessive epistasis governing resistance to *M. phaseolina*. However, variability among populations of *M. phaseolina* may result in such resistance being short-lived (Hernández-Delgado, 2009; Gupta *et al*., 2012; Iqbal & Mukhtar, 2014). The high genetic diversity exhibited by *M. phaseolina* is reflected not only in the numbers of genetically unique isolates obtained from a single host plant or a geographical location, but also from distinct hosts and geographical origins (Babu et al., 2010; Sarr *et al*., 2014). In addition, isolates from a single host species may vary in pathogenicity on different cultivars of that species, suggesting the existence of distinct physiological races of *M. phaseolina* (Sulaiman & Patil, 1966; Mahdizadeh *et al*., 2011).

Although the pathogen exhibits a high degree of morphological, pathogenic, physiological and genetic variation, no sexual stage has been identified (Almeida *et al*., 2003; Crous *et al*., 2006). Filamentous fungi lacking a sexual cycle must rely on gene mutations, hyphal fusion and mitotic recombination to generate genetic variation (Boddy, 2015; Carlile, 1986). Genetic variability caused by mitotic recombination was first described by Pontecorvo *et al.* (1953) using the term parasexuality to define the processes resulting in recombinant strains that had not passed through the sexual cycle.

The parasexual cycle starts with the formation of the heterokaryon, a hypha containing nuclei of two distinct genetic types in a common cytoplasm. Heterokaryotic hyphae are formed by the anastomosis of the two homokaryotic hyphae. The nuclei within the heterokaryotic hyphae may fuse, producing heterozygotic diploid nuclei (Leslie, 1993; Strom & Bushly, 2016).

The heterokaryon's viability depends on the genetic characteristics of the *het* (heterokaryon incompatibility) or *vic* (vegetative incompatibility) loci. Only genetically identical strains for a set of *het* or *vic* loci form stable heterokaryons and constitute members of the same vegetative compatibility group (VCG) (Leslie, 1993; Saupe, 2000). Strains carrying gene mutations that prevent them from fusing to form heterokaryons, even among themselves, have been identified in several fungal species and this trait is termed as heterokaryon self-incompatible (HSI) (Glass et al., 2000). Previous research has demonstrated that Brazilian populations of *M. phaseolina* have high genetic and pathogenic variability (Almeida *et al*., 2003; Claudino & Soares, 2014). Thus the aim of this study was to (*i*) assess the diversity of vegetative compatibility reactions among isolates of *M. phaseolina* derived from different host plants and geographical areas in Brazil; (*ii*) identify whether the heterokaryon self-incompatible trait is present in isolates of *M. phaseolina*; and (*iii*) obtain heterozygous diploid nuclei in order to demonstrate the potential for the occurrence of a parasexual cycle in *M. phaseolina*.

Materials and methods

M. phaseolina isolates and media

Host, geographical location, source and GeneBank accession numbers of the isolates of *M. phaseolina* used in the current study are shown in Table 1. The media used to maintain the fungus, and in generation of mutants were as follows (Castro-Prado *et al*., 2007): basal medium (BM; 1000mL distilled H_2O, 30 g saccharose, 1 g KH_2PO_4, 0.5 g $MgSO_4.7\ H_2O$, 0.5 g KCl, 10 mg $FeSO_4.7\ H_2O$, 15 g agar, and 0.2 mL trace element solution [95 mL distilled H_2O, 5 g citric acid, 1 g $Fe(NH_4)_2(SO_4)_2.6\ H_2O$, 0.25 g $CuSO_4.5\ H_2O$, 50 mg $MnSO_4.H_2O$, 50 mg H_3BO_3, and 50 mg $Na_2MoO_4.2\ H_2O$]); complete medium (CM; Czapeck-Dox, 1000 mL distilled H2O, 10 g glucose, 2 g peptone, 0.5 g yeast extract, 1 g hydrolyzed 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, and 2 µg biotin). Cultures were maintained on at 5ºC in amber flasks.

Genomic DNA extraction

Pure cultures of *M. phaseolina* were grown in CM for 5 days 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 White *et al*. (1990).

PCR using MpKF1 and MpKR1

The species-specific primers MpKF1 (5'-CCGCCAGAGGACTATCAAAC-3') and MpKR1 (5'-CGTCCGAAGCGAGGTGTATT-3') were synthesised and amplified a 350 bp product of the ITS region of the ribosomal DNA (rDNA) gene (Babu *et al*., 2007). PCR amplification was performed in a 25 µl reaction mixture containing 50 ng of DNA template; 200 µM of each of dATP, dCPT, dGTP and dTTP; 50 mM KCl; 10 mM Tris-HCl (pH 8.0); 1.5 mM $MgCl_2$; 1 µM MpKF1 primer; 1 µM MpKR1 primer; 2 U of Platinum® Taq DNA polymerase (Invitrogen, Life Technologies, Brazil). Initial denaturation of 94°C for 5 min was followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing (59°C, for 2 min) and extension (72°C, for 2 min). A final extension of 72°C for 5 min was incorporated into the program. The tubes were held at 4°C until recovery of the samples. All amplified DNA products were checked for presence and quality by electrophoresis on agarose gels (1.0%), stained with ethidium bromide (Invitrogen, Carlsbad, CA, USA) and photodocumented under UV light. All experiments were repeated twice.

PCR and ITS sequencing

The ITS regions 1 and 2 of the 5.8S rRNA gene of the isolates of *M. phaseolina* were amplified using the forward primers ITS1, ITS5 and the reverse primer ITS4 (White *et al*., 1990, Sarr et al.,

2014). The PCR products were subject to electrophoresis on a 1.0% agarose gel stained with ethidium bromide and photodocumented under UV light. The PCR products of isolates 1 to 8 were purified and sequenced by Macrogen Inc., South Korea, and those of isolates 9 to 13 were purified and sequenced by the Institute of Biosciences (University of São Paulo, São Paulo Brazil). The sequences from all isolates were compared with the sequence of the ex-epitype culture of *M. phaseolina* (isolate CBS 205.47, GenBank accession KF951622) using a BLASTn search.

Isolation of *nit* mutants

Mycelial plugs (5 mm) were removed from the edges of colonies of *M. phaseolina* cultivated on CM and transferred to Petri plates with BM + $NaNO_3$ (2 g/L) + $KClO_3$ (35 to 40 g/L). A total of fifteen plates were prepared for each isolate, which were maintained at 28°C, for 12-15 days. Fast-growing sectors emerging from the restricted colonies 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 purified and transferred to Petri plates containing BM supplemented with different nitrogen sources: sodium nitrate (2 g/L), potassium nitrite (0.85 g/L), hypoxanthine (0.1 g/L) and ammonium tartrate (0.92 g/L). Plates were maintained at 28°C, for 6 to 10 days, and *nit* mutants were phenotypically characterized based on their ability to metabolize the different nitrogen sources (Beever and Parkes, 2003).

Obtaining heterokaryons and diploids

Mycelial plugs (5 mm in diameter) of complementary *nit* mutants were paired equidistant (approximately 1.0 cm) on BM + $NaNO_3$ (2 g/L) in Petri plates. After 6 to 12 days of incubation at 28ºC, the plates were visually inspected for prototrophic heterokaryotic growth in the contact area of the paired mutants. To demonstrate the need for physical contact between the hyphae of the pairing

mutants for heterokaryon formation, a sterilized dialysis membrane was placed between the two mutant colonies at the interaction zone. All experiments were repeated twice. Heterokaryons growing on BM + $NaNO_3$ may produce: (*a*) auxotrophic segregants exhibiting the *nit* phenotype of the paired mutants, and (*b*) fast-growing sectors, exhibiting the *nit*+ phenotype with a growth rate similar to the original wild isolate. Such prototrophic fast-growing sectors have the properties of unstable diploids (Strom and Bushly, 2016).

Phenotypic analysis of auxotrophic segregants from heterokaryon and diploid colonies

Mycelial plugs (5 mm in diameter) of mitotic segregants (or sectors) of *nit* phenotype were obtained from heterokaryon and diploid colonies grown on BM + $NaNO_3$. Segregants were transferred to BM supplemented with different nitrogen sources (as described above, sodium nitrate, potassium nitrite and hypoxanthine) for their phenotypic characterization. Experiments were repeated twice.

Vegetative compatibility grouping

The formation of a viable heterokaryon between two complementary *nit* mutants assigned their wild parent isolates, which were designated heterokaryon self-compatible strains (HSC), to the same vegetative compatibility group (VCG). Lack of complementation between the two complementary *nit* mutants may mean: (*a*) their original wild isolates are in different VCGs, or (*b*) one or both of their wild isolates are unable to form a heterokaryon in which complementation can occur. Such isolates are designated heterokaryon self-incompatible (HSI) (Rosada *et al*., 2013).

Hyphal anastomoses

Hyphal fusions of specific isolates were examined using a compound light microscopic (Olympus, Optical Co). Fresh cultures were established by mycelial transfer from a 5-6 day old culture grown on BM + $NaNO_3$ to the centre of a 90 × 15 mm Petri plate containing 12 ml 3% water agar. After 5 days of incubation at 30º C, 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. The number of hyphal fusions was 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). The resulting data for the three discs/ isolate were analysed by analysis of variance (ANOVA) followed by a post hoc Bonferroni means separation ($\alpha = 0.05$) (Graphpad Prism software Version 6.0, San Diego, USA). Standard deviations of the means were calculated.

Results

Taxon-specific PCR and ITS sequencing

The ITS sequences from isolates 1 to 13 showed 99-100% homology with the GenBank accession KF951622.1, the sequence of the ex-epitype culture (isolate CBS 205.47) of *M. phaseolina*. All isolates that were tested produced a 350-bp fragment, following PCR with primers MpKF1 and MpKR1 and gel electrophoresis (Fig. 1), as described by Babu *et al*. (2007).

Isolation of *nit* mutants and heterokaryon

A total of four hundred and seventy-six nitrate non-utilizing mutants were recovered from the 13 wild-type isolates of *M. phaseolina*. The mutants produced thin and sparse colonies on BM containing only sodium nitrate as a nitrogen source and were phenotypically classified as: *Nit1* (mutants for the nitrate reductase structural gene), *Nit3* (mutants for the regulatory gene of nitrate and nitrite reductase) and *NitM* (mutants in loci codifying the molybdenum-containing cofactor necessary for nitrate reductase activity) (Fig. 2). For each of the 13 wild-type isolates of *M. phaseolina*, two or three types of *nit* mutants were recovered, with *Nit1* as the predominant phenotypic class for most (61.5%) isolates (Table 2).

When two complementary *nit* mutants from the same or distinct isolates were paired on BM + $NaNO_3$, prototrophic growth was observed at the junction of the colonies of the paired mutants (Fig. S1a). Such prototrophic growth could result from: (*a*) reversion to prototrophy; or (*b*) heterokaryosis. If one or both of the paired mutants had undergone reversion to prototrophy, the recovery of the auxotrophic markers, originally present in the paired mutants, would not be observed. In our analyses, when mycelial plugs were removed from the prototrophicaly growing regions and transferred to BM + $NaNO_3$, auxotrophic segregants exhibiting the auxotrophies of the paired mutants were obtained (Table 3, Fig. S1b). Thus, the prototrophic growth resulting from the pairing of two complementary *nit* mutants is not due to reversion to prototrophy, but it is due to heterokaryosis. In addition, the prototrophic growth did not form where mutant pairs were separated by sterilized dialysis membrane (Fig. S1c), demonstrating that physical contact between the complementary mutants is necessary for prototrophic growth to occur.

Diploid formation

The heterokaryon formed by the pairing of complementary *nit* mutants from isolate 6 (*Nit3/Nit1*) produced a fast-grown sector with a prototrophic *nit*+ phenotype when growing on BM + $NaNO_3$ (Fig. S1d). Whereas the heterokaryotic colonies are macroscopically compact, irregular and slow-growing, the fast-growing sector, designated D653, after purification in BM + $NaNO_3$, showed colony morphology distinct from the heterokaryon and formed colonies with regular edges and homogeneous growth, with growth rate similar to the original wild-type isolate 6. The colonies of D653 showed mitotic instability, producing spontaneously mitotic segregants which exhibited the *nit* phenotypes of the original paired mutants (Table 3, Fig. S1e). This was also observed when D653 was growing in the presence of the haploidizing agent benomyl (0.5 μg/ml) (Fig. S1f).

Characterization of the VCGs and the HSC and HSI phenotypes

Auxotrophic *nit* mutants from the same isolate were paired on BM + $NaNO_3$ to determine whether they were HSC or HSI mutants. All isolates, except isolate 1, were characterized as HSC due to the formation of a dense heterokaryon where hyphae of the two colonies made contact. By pairing complementary *nit* mutants of the HSC isolates in all possible combinations on BM + $NaNO_3$, ten distinct VCGs were identified, with eight of these VCGs consisting of a single isolate (VCGs A-G, I), and two consisting of two isolates (VCGs H and J) (Table 2). Isolates 2 to 4, which were obtained from a single municipality, but from different farms, were in VCGs A to C. Isolates 5-7, which grouped in VCGs D, E and F were obtained from the same host species but from distinct locations. On the other hand, isolates 8 (VCG G) and 6 (VCG E), although obtained from the same municipality, were isolated from two different farms. Isolates 9 to 13, all collected from *V. unguiculata*, were grouped in 3 different VCGs. Isolate 11 formed a distinct VCG and was from a different geographic location and host genotype (Table 1). Isolates 12 and 13 grouped in the same VCG (J), and were from the same host species and location. In contrast, isolates 9 and 10, which were obtained from different geographical locations and different host genotypes, grouped in the same VCG (H).

No intra- or inter-isolate complementation was observed for isolate 1 when its complementary *nit* mutants were paired with each other or with complementary *nit* mutants of distinct isolates. Moreover, examination of hyphal fusions of isolates 1, 4 (HSC) and 13 (HSC) under a light microscope showed that the average number of hyphal fusions/mm^2 for isolate 1 (5.73 ± 1.614) was significantly ($P < 0.0001$) fewer when compared with the average number of hyphal fusions/mm^2 produced by isolates 4 and 13, (37.33 ± 4.177 and 33.67 ± 2.028, respectively). Results led to the characterization of isolate 1 as HSI (Fig. 3). Due to its HSI phenotype, it could not be classified in any of the vegetative complementation groups identified using *nit* mutants of the other isolates (Table 2).

Discussion

The results of this research show, for the first time, that vegetatively compatible isolates of *M. phaseolina* may form viable heterokaryons and unstable diploid. A heterozygous diploid sector was obtained spontaneously from a heterokaryon formed between complementary *nit* mutants of isolate 6. Diploid sector was probably formed by the fusion of two genetically complementary nuclei within the heterokaryotic mycelium (Roper *et al*., 2013). The mitotic propagation of the diploid strain produced, by haploidization, auxotrophic segregants with the same phenotypes of the original paired mutants. Thus, our results demonstrate that the parasexual cycle can occur in *M. phaseolina*.

Pairings of complementary *nit* mutants allowed us to classify 12 of the 13 isolates of *M. phaseolina* in ten VCGs. Since strains belonging to the same VCG share the same haplotype with respect to the *vic* or *het* genes, the genotype diversity of a sample can be represented by the ratio of the number of haplotypes to sample size (Caten & Newton, 2000). We found 10 distinct VGCs among the 12 isolates of *M. phaseolina*, in the sample, resulting in an estimated diversity of 0.83. This result demonstrates the high variability of the isolates examined in the current study. The high number of VCGs observed can be partially explained by the restricted dispersal ability of *M. phaseolina*. Since this pathogen is a soil-inhabiting fungus, and usually infects the below-ground portions of its hosts, its ability to be dispersed between growing areas, or even within an area, is limited compared to most aerially dispersed pathogens. Consequently, the genetic flow between distinct subpopulations is restricted. On the other hand, since the pathogen is also seedborne, it does have occasion for long distance dispersal, meaning that geographically distinct sites can have compatible strains. This paradox may explain the degrees of genetic variability reported in the literature for *M. phaseolina* (Su *et al*., 2001; Almeida *et al.*, 2003; Gupta *et al*., 2012).

The results of the VCG analysis demonstrated that the geographical isolation had a substantial effect on the VCGs observed among isolates of *M. phaseolina* in Brazil. Except for the isolates 9 and 10, grouped in the same VCG irrespective of their different geographic origins, most of the

isolates analysed in this study were obtained from distinct locations and were allocated in distinct VCGs. Similar results were previously observed in the phytopathogens *Colletotrichum truncatum* and *C. acutatum*. Whereas in *C. truncatum*, the isolates B and E, obtained respectively from the Brazilian States of Paraná and Minas Gerais, were allocated in distinct VCGs (Sant'Anna *et al*., 2010); in *C. acutatum*, isolates from different locations and hosts (strawberry and Tahiti lime) were assigned on the same VCG (Franco *et al*., 2011).

Vegetative compatibility analysis is a very useful tool for analysing pathogenic and non-pathogenic fungal populations and for identifying new races in crop production areas or regions or among host genotypes (Mpofu & Rashid 2001; Krnjaja *et al*., 2013). Since vegetatively compatible isolates have the same allele at each and every incompatibility locus, isolates of similar pathogenicity may belong to the same VCG (Dervis *et al*., 2009; Krnjaja et al., 2013). Therefore, in plant disease diagnosis, the pairing of an unknown pathogenic fungus with a tester strain belonging to a known VCG provides a faster and simpler way to determine the identity of the infecting fungus compared to traditional pathogenicity tests using standard differential host species or cultivars (Leslie, 1993). Further work is needed to understand these and other traits in relation to VCGs in *M. phaseolina.*

Since the formation of stable heterokaryons among compatibles isolates only occurs after cell fusion, isolates carrying mutations involved in the anastomosis process will not form heterokaryons with any other isolates, not even with themselves (Glass *et al*., 2000). In our analysis, the inability of isolate 1 to forming viable heterokaryons between its genetically complementary *nit* mutants and its reduced count of hyphal fusions compared with its HSC counterparts (isolates 4 and 13) led us to characterize isolate 1 as having an HSI phenotype. Although statistically non-significant variations in the number of hyphae anastomoses were observed among HSC isolates 4 and 13 of *M. phaseolina*, significant variations in the frequency of hyphal anastomosis among HSC isolates of *Aspergillus flavus* and *Fusarium solani* have been previously demonstrated (Hawthorne & Rees-

George, 1996; Rosada *et al*., 2013). We found that hyphal anastomosis in HSI isolate 1, under laboratory conditions, was on average, about six times less common when compared to HSC isolates 4 and 13. Similar results were observed for HSI isolates of *F. solani* which produced about four times less hyphal anastomosis when compared to HSC isolates (Hawthorne & Rees-George, 1996).

Although haploid recombinants were not obtained after haploidization of the diploid D653, the ability of *M. phaseolina* to form heterozygous diploid nuclei suggests that this plant pathogen may develop undesirable genetic changes during continuous asexual propagation. The fact that no sexual stage has been identified for *M. phaseolina* (Crous *et al*., 2006), we hypothesize that the extensive genetic variability (Mahdizadeh *et al.*, 2011) of this plant pathogen may be in part due to the occurrence of anastomosis and heterokaryosis among compatible isolates. The vegetative compatibility observed between some of the isolates derived from *V. unguiculata* suggests that they are potentially able to exchange genetic material. This feature, allied to the seed-borne nature of *M. phaseolina* result in occasional opportunity for long-distance dispersal, which raises concerns regarding management of charcoal rot. Exchange of genetic information could modify the pathogenicity of isolates, resulting in novel pathotypes adapted to previously resistant host varieties.

The results of this research provide the first evidence of the parasexual cycle, vegetative compatibility reactions and the HSI phenotype in *M. phaseolina.* Since each vegetative compatibility group represents a genetically isolated subpopulation in the absence of a sexual stage (Kalc Wright *et al*., 1996), VCG determination may reflect genetic similarities among different isolates of a species. This way, VCGs may be ideally suited for the production of profiles of populations in *M. phaseolina*.

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Conflicts of interest

The authors report no conflicts of interest

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Figure legends

Fig. 1. Amplification products of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) gene as taxon-specific identification of *Macrophomina phaseolina* (primer MpKF1 in combination with primer MpKR1). Lane M, molecular marker; lane N, negative control; lanes 1 to 13, *M. phaseolina* isolates, respectively from *Sesamum indicum* (lane 1), *Ricinus communis* (lanes 2 to 7), *Glicine max* (lane 8) and *Vigna unguiculata* (lanes 9 to 13). Agarose gel stained with ethidium bromide and visualized under ultra-violet light.

Fig. 2. Phenotypic characterization of *nit* mutants. (a) wild type isolate growing in BM + $NaNO_3$. Auxotrophic *nit* mutants growing in MB + sodium nitrate (b), MB + potassium nitrite (c) and MB + hypoxanthine (d). 1, 2, 3 and 6: mutants characterized as *Nit3*. 4, 7, 8 and 9: mutants characterized as *NitM*. 5: mutant characterized as *Nit1*.

Fig. 3. Mean number of hyphal fusions/mm^2 of isolates HSC (4 and 13) and HSI (1). Column heights indicate means ± standard deviations of three experiments. The asterisk (*) denotes a statistical difference between the two HSC isolates (4 and 13) and HSI (1). No significant difference is denoted by (^{a}). Data were analysed by ANOVA followed by a post hoc Bonferroni means separation ($\alpha = 0.05$).

Supplementary figure legend

Fig. S1 Heterokaryon and diploid formation between complementary *nit* mutants of *M. phaseolina.* (a) Heterokaryon (het) formed with mutants *Nit1* and *Nit3* from isolate 7. (b) Heterokaryotic colony formed by complementary *nit* mutants from isolate 7 showing mitotic segregation of the crossed *nit* mutants (arrows). (c) Pairing between *Nit3* and *Nit1* mutants from isolate 7, separated by a sterilized dialysis membrane. Heterokaryons (*het*) did not occur beneath the dialysis membrane which impairs direct contact between the pairing mycelia. (d) Heterokaryotic colony formed by complementary *nit* mutants from isolate 6 showing a fast-growing sector (arrow). (e) Diploid D653 growing on complete medium. Arrows indicate the auxotrophic *nit* segregants spontaneously produced by D653. (f) Diploid D653 producing auxotrophic *nit* segregants (arrows) when growing in basal medium + NaNO3 + benomyl (0.5 μg/ml). Bar = 5.0 mm.

Table 1. Host, geographical origin, source and GeneBank accession numbers of the isolates of *Macrophomina phaseolina* included in the current study.

Isolate Number	Host		Geographical Location	Source		GenBank accession numbers
	Species	Genotype/Variety	(municipality/state)	Culture Collection	Original Code	ITS
1	*Sesamum indicum*	BRS Seda	Campina Grande/PB	Embrapa Algodão	CCMF-CNPA 274	KU058926
2	*Ricinus communis*	BRS Energia	Irecê/BA	Embrapa Algodão	CCMF-CNPA 283	KU058954
3	*Ricinus communis*	BRS Paraguaçu	Irecê/BA	Embrapa Algodão	CCMF-CNPA 290	KU058938
4	*Ricinus communis*	BRS Nordestina	Irecê/BA	Embrapa Algodão	CCMF-CNPA 292	KU058940
5	*Ricinus communis*	BRS Energia	Campina Grande/PB	Embrapa Algodão	CCMF-CNPA 296	KU058944
6	*Ricinus communis*	*	Balsas/MA	Embrapa Algodão	CCMF-CNPA 653	KU058947
7	*Ricinus communis*	*	Tasso Fragoso/MA	Embrapa Algodão	CCMF-CNPA 654	KU058948
8	*Glycine max*	unknown	Balsas/MA	Embrapa Algodão	CCMF-CNPA 707	KU058952
9	*Vignia unguiculata*	EAV 201	Teresina/PI	Embrapa TC	MP 16	MF563940
10	*Vignia unguiculata*	Sempre Verde	Valença do Piauí/PI	Embrapa TC	MP 18	MF563941
11	*Vignia unguiculata*	BRS Guariba	Uruçuí/PI	Embrapa TC	MP 21	MF563942
12	*Vignia unguiculata*	IPA 207	Teresina/PI	UFRPE	CMM-2107	MF563943
13	*Vignia unguiculata*	IPA 207	Teresina/PI	UFRPE	CMM-2111	MF563944

*distinct advanced breeding lines of Evofuel Company. PB, BA, MA, PI = Paraíba, Bahia, Maranhão and Piaui States, Brazil. Embrapa TC = Embrapa Tabuleiros Costeiros. UFRPE = Universidade Federal Rural de Pernambuco (Brasil).

Table 2. The frequency of *nit* mutants and vegetative compatibility groups (VCG) among Brazilian isolates of *Macrophomina phaseolina* collected from different geographical locations and hosts.

Isolates	**Frequency (%) of auxotrophic *nit* mutants**			VCG
	nit1	*Nit3*	*NitM*	
1	88.7	11.3	00	n.a.*
2	30.8	69.2	00	A
3	40	20	40	B
4	44.5	22.2	33.3	C
5	7.2	79.7	13.1	D
6	92.3	7.7	00	E
7	38.7	41.9	19.4	F
8	80	6.7	13.3	G
9	80	20	00	H
10	77.3	22.7	00	H
11	54.2	42	3.8	I
12	83.3	16.7	00	J
13	38.5	38.5	23	J

(*) not analysed.

Table 3. Phenotypic segregation of heterokaryons and diploid colonies formed with *nit* mutants of *Macrophomina phaseolina*.

Pairing of *nit* mutants		Phenotypes of mitotic (parasexual) segregants		
Paternal 1[a]	Paternal 2[a]	Paternal 1	Paternal 2	Total
9 (*Nit1*)	10 (*Nit3*)[b]	11	8	19
12 (*Nit3*)	13 (*NitM*)[b]	25	19	44
3 (*Nit1*)	3 (*NitM*)[c]	19	10	29
D653 (diploid segregant)		Paternal 1	Paternal 2	Total
6 (*Nit3*)	6(*Nit1*)[c]	10	36	46

[a]Paternal 1 and [a]Paternal 2: auxotrophic *nit* mutants obtained from wild isolates and used to form heterokaryons. [b]Inter-isolates pairing of *nit* mutants. [c]Intra-isolate pairing of *nit* mutants.