Eur J Plant Pathol
https://doi.org/10.1007/s10658-022-02530-w

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

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Accepted: 25 May 2022

Abstract Charcoal rot is an economically important fungal disease of economically important crops. Several species of the *Macrophomina* genus, such as *M. pseudophaseolina* and *M. 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 the 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

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Introduction

The genus *Macrophomina* is currently recognized as a member of the family Botryosphaeriaceae that includes the species *Modiolula phaseolina* (Tassi) Goid.,

Published online: 31 May 2022

M. pseudophaseolina Crous, M.P. Sarr & Ndiaye, *M. euphorbiicola* A.R. Machado, D.J. Soares & O.L. Pereira, *M. vaccinii* Zhang ter & Zhao, and *M. 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 can be highly effective and causes 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., 2019; 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 & 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.

Materials and methods

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; 1000 mL distilled H_2O, 30 g saccharose, 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, 1000 mL 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 5 °C in amber flasks.

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).

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 94 °C; 5 cycles of 3 min at 94 °C; 3 min at 35 °C and 2 min at 72 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C, 90 sec at 72 °C, and a final extension at 72 °C 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 photo-documentation equipment (UVP GDS-8000 System). Only high-intensity bands reproducible in two independent amplification reactions were included in the data analyses.

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 the arithmetic average (UPGMA) algorithm. The dendrogram's cut-off value was based on the method described by Jamshidi and Jamshidi (2011).

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*

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	KU058920
5	*A. hypogaea*	Apodí/RN	*M. pseudophaseolina*	Embrapa Algodão	CCMF-CNPA 668	KU058919
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
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	*Boerhavia 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

Isolates 1–6 and 28–30 were provided by Dr. Dartanhã J. Soares. Isolates 9–27 were kindly donated by Dr. Rui S. Júnior

[a] BA, RN, CE, PB Bahia, Rio Grande do Norte, Ceará and Paraíba States, Brazil. UFERSA = Universidade Federal Rural do Semi-Árido, Mossoró-RN, Brazil

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 6–10 days of 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).

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 28 °C. 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, produce 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.

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 30 °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. 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.

Results

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 ± 0.15) and 5

Table 2 Number (No) 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	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
16	32	84.2	0	0	0	0	6	15.8	H	HSC
17	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	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

(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. 1). 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).

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).

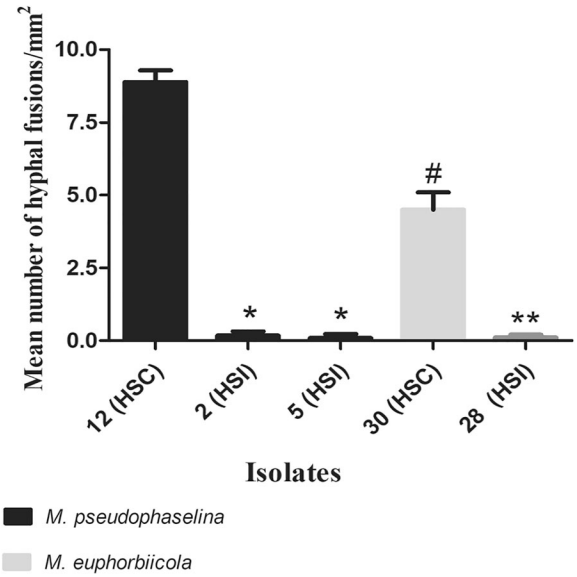

Fig. 1 Mean number of hyphal fusions/mm^2 of the HSC isolates 12 (*M. pseudophaseolina*) and 30 (*M. euphorbiicola*), and of the HSI isolates 2, 5 (*M. pseudophaseolina*) and 28 (*M. euphorbiicola*). Column heights indicate means ± standard deviations of three experiments. (*) denotes statistical differences 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 ($p < 0.0001$)

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 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.

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 recombinants was demonstrated by their mitotic stability when growing in BM + $NaNO_3$ + benomyl, without the production of new mitotic sectors (results not shown).

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

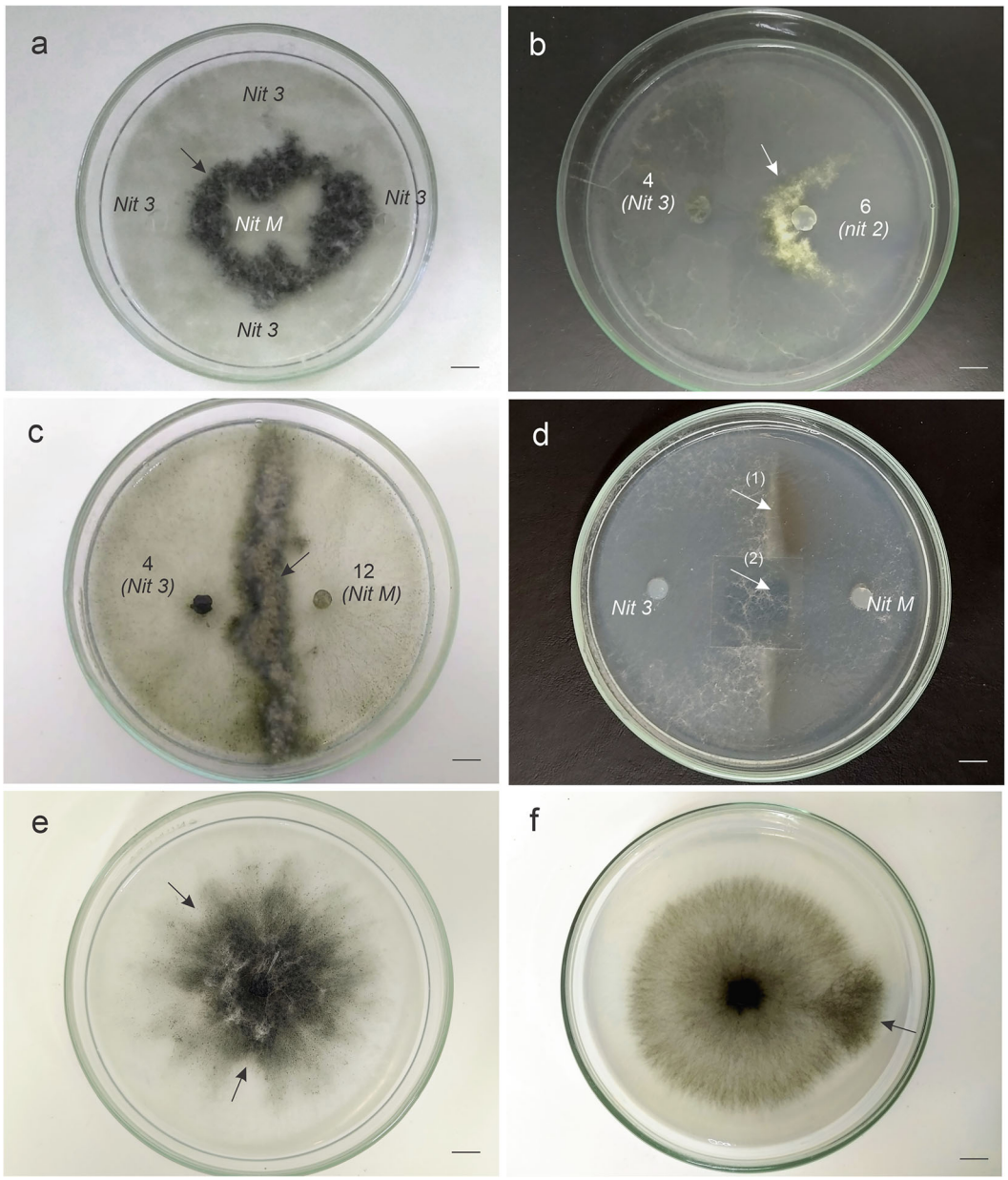

Fig. 2 Heterokaryotic and diploid colonies formed between complementary *nit* mutants of *M. euphorbiicola* and *M. pseudophaseolina* isolates. (a) Heterokaryon (arrow) formed by complementary *nit* mutants from isolate 30 of *M. euphorbiicola*; (b) Heterokaryon (arrow) formed by isolates 4 and 6 (VCG C) in BM + $NaNO_3$ after 16 days of incubation at 28 °C; (c) Heterokaryon (arrow) formed by isolates 4 and 12 (VCG D) in BM + $NaNO_3$ after 8 days of incubation at 28 °C; (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 28 °C. Colony shows mitotic instability (arrows) and irregular edges; (f) Diploid colony (D4//6) growing in BM + NaNO3 after 3 days of incubation at 28 °C. Colony, showing homogeneous morphology and regular edges, produced a parasexual prototrophic recombinant (*nit*+) named R2 (arrow). Bar = 5.0 mm

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

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* (Fig. 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 (Fig. 3b). Results demonstrated that RAPD analysis was useful to distinguish genomic differences among *M. pseudophaseolina* diploid, heterokaryotic, and prototrophic recombinant colonies.

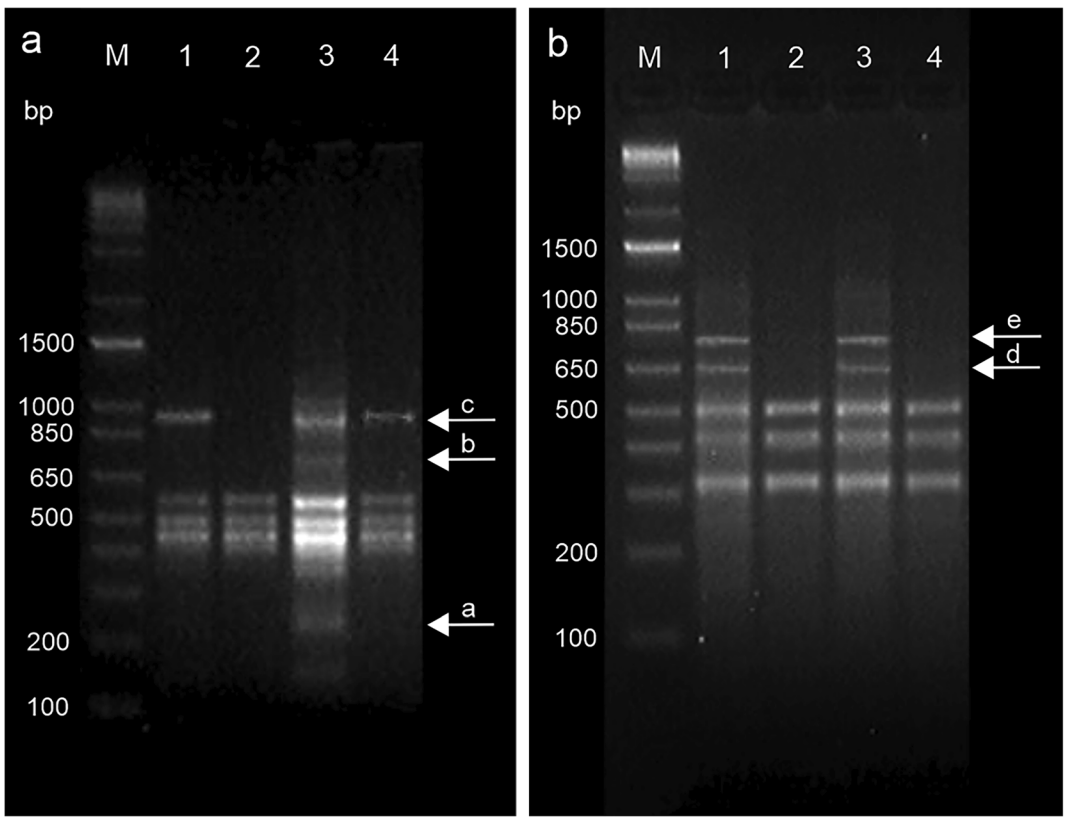

Fig. 3 RAPD profiles of isolate 14, diploid D14/14, heterokaryon *nit1*/*Nit3*, and recombinant R1 (lines 1, 2, 3, 4) in agarose gel using primers OPW-07 (a) and OPW-09 (b). Column M = molecular weight DNA ladder in bp (100–1500), bp = base pairs. Arrows indicate polymorphic bands of 250 bp (a), 700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

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 (Fig. S1, 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% 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 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 58% in relation to Group I (Fig. 4). *Macrophomina 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% 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).

Discussion

The current study demonstrates the occurrence of vegetative compatibility reactions and HSI 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* (Correll 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.,

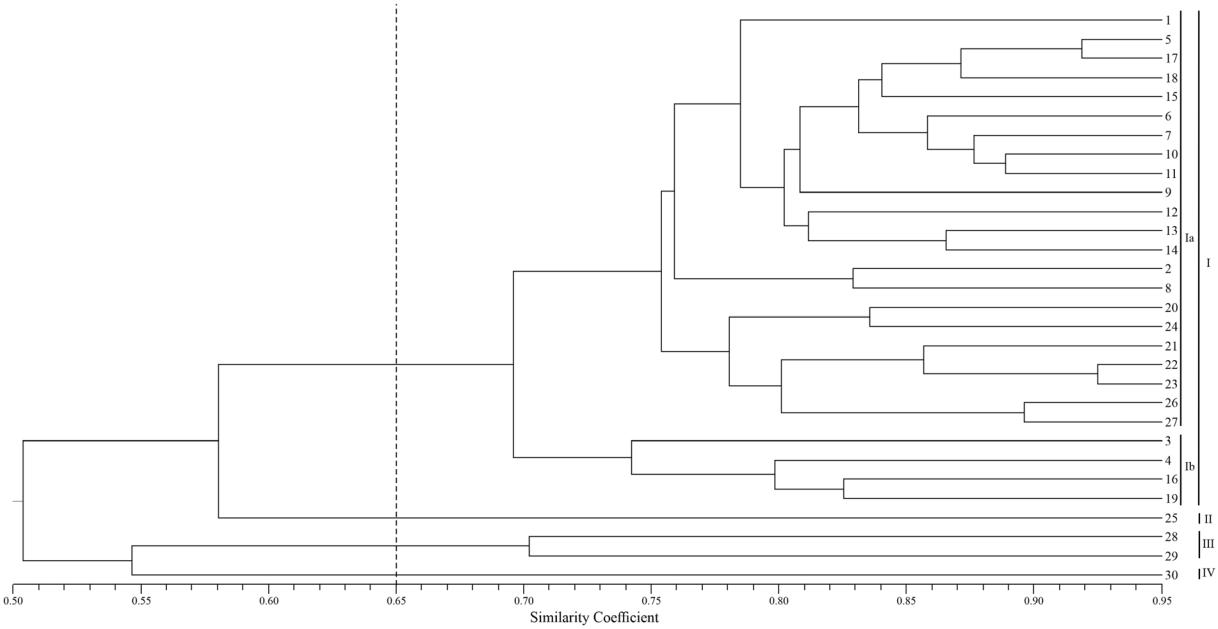

Fig. 4 Dendrogram (NTSYS software) constructed with UPGMA clustering method from *M. pseudophaseolina* (totaling 27 isolates) and *M. euphorbiicola* isolates (totaling 3 isolates), using 9 different primers and RAPD analysis. Similarities were computed from 136 random polymorphic bands. I to IV = RAPD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

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, and variations, statistically significant in the frequency of hyphal anastomosis among HSC isolates, have been previously described in *A. flavus* and *F. 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 a 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, F. oxysporum* f. sp. *radicis-lycopersici*, and *Verticillium dahlia*, may exchange genetic information between different VCGs, since the complete genetic isolation among these VCGs has not yet developed (Rodríguez-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 the 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 analysed (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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-022-02530-w.

Acknowledgements The authors would like to thank Dr. Rui Sales Júnior for kindly sending the isolates 9–27.

Availability of data and material Fungal isolates are available from Brazilian culture collections.

Code availability Not applicable.

Authors' contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Priscila A. Sybuia, Gabriela de Castro-Prado Carlos A. Zanutto, Edilson N. Kaneshima, and Marialba A. A. de Castro-Prado. The first draft of the manuscript was written by Marialba A. A. Castro-Prado and all authors commented on previous versions of the manuscript. All authors read and approved the manuscript and consented for publication in the European Journal of Plant Pathology.

Funding The current research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). P.A.S. is a holder of CAPES fellowship.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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