

Genetic diversity of *Macrophomina phaseolina* and antifungal potential of lemongrass essential oil

Diversidade genética de *Macrophomina phaseolina* e potencial antifúngico do óleo essencial de capim-limão

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ABSTRACT - *Macrophomina phaseolina* is a soil-borne fungal pathogen responsible for root rot and vine decline in melons. To develop effective control strategies, it is essential to understand the pathogen's genetic diversity. This study aimed to characterize the genetic diversity of *M. phaseolina* isolates and evaluate the effectiveness of *Cymbopogon flexuosus* (lemongrass) essential oil as a control agent. DNA from 30 isolates was extracted and analyzed using 13 RAPD markers, which generated 135 polymorphic bands (71.85% polymorphism). Genetic similarity among isolates ranged from 0.23 to 0.92. To test the antifungal potential of *C. flexuosus* oil, two specific isolates (CMM-4739 and CMM-4762) were treated with six oil concentrations (0.02, 0.05, 0.10, 0.15, 0.20, and 0.25%). A negative control (PDA medium) and a positive control (*Maxim fungicide*) were included. Colony diameter was measured daily in two perpendicular directions; the control treatment reached the edge of the Petri dish within three days. Results showed that a concentration of 0.15% resulted in the complete *in vitro* growth inhibition of both isolates. No significant difference in oil efficacy was observed between the two isolates. This study concludes that RAPD markers are suitable for assessing the genetic diversity of *M. phaseolina* and that *C. flexuosus* essential oil is highly effective at inhibiting fungal growth *in vitro*. These findings suggest that *C. flexuosus* oil holds significant potential for future *in vivo* management of the *M. phaseolina*-melon pathosystem.

RESUMO - *Macrophomina phaseolina* é um fungo habitante de solo, agente causal da podridão radicular e declínio de ramas em meloeiro. Para o desenvolvimento de métodos de controle, é importante conhecer sua diversidade genética. Assim, este trabalho teve como objetivo caracterizar a diversidade genética de *M. phaseolina* e avaliar a eficácia do óleo essencial de *Cymbopogon flexuosus* em seu controle. DNAs de 30 isolados de *M. phaseolina* foram extraídos e analisados com 13 marcadores RAPD. Os marcadores geraram um total de 135 bandas (71,85% polimórficas). A similaridade genética variou de 0,23 a 0,92. Para o ensaio do óleo essencial de *C. flexuosus*, dois isolados de *Macrophomina* (CMM-4739 e CMM-4762) foram utilizados em seis doses do óleo (0,02; 0,05; 0,10; 0,15; 0,20 e 0,25%). Um controle negativo (meio BDA) e um controle positivo (fungicida Maxim) foram incluídos. Medições do diâmetro das colônias foram realizadas diariamente em duas direções perpendiculares. Na concentração de 0,15%, houve inibição completa do crescimento *in vitro* dos isolados. Não houve diferença no efeito do óleo essencial entre os isolados estudados. Este estudo conclui que o marcador RAPD foi adequado para avaliar a diversidade genética de *M. phaseolina* e que o óleo essencial de *C. flexuosus* demonstrou eficácia na inibição do crescimento *in vitro* dos isolados estudados. Esses resultados sugerem que o RAPD pode ser usado para demonstrar a diversidade genética de *M. phaseolina* e que o óleo essencial de *C. flexuosus* tem potencial para futuros estudos *in vivo* para o manejo do patossistema de *M. phaseolina* e melão.

Keywords: *Cymbopogon flexuosus*. RAPD-PCR. Root rot and vine decline. Soil-borne fungi.

Palavras-chave: *Cymbopogon flexuosus*. RAPD-PCR. Podridão radicular e declínio da videira. Fungos de solo.

Conflict of interest: The authors declare no conflict of interest related to the publication of this manuscript.



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Received for publication in: September 15, 2025.
Accepted in: April 10, 2026.

Editor in Chief: Aurélio Paes Barros Júnior

Data Availability: The data that support the findings of this study can be made available, upon reasonable request, from the corresponding author.

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INTRODUCTION

Melon (*Cucumis melo* L.) is a member of the Cucurbitaceae family and holds significant economic importance in Brazil. In 2023, Brazilian production reached 862,387 tons across 30,535 hectares, generating a production value of R\$ 1.25 billion. The state of Rio Grande do Norte is the leading producer, accounting for R\$ 823 million of this total (IBGE, 2024).

However, various phytosanitary challenges threaten melon growth, development, and yield. Notably, certain soil-borne pathogens cause charcoal rot in stems and roots, inducing a water imbalance between the roots and shoots, particularly near harvest. Several etiological agents are associated with this disease, primarily fungi within the genus *Macrophomina* (NEGREIROS et al., 2019). While *M. phaseolina* was the only known species in the genus until 2013, subsequent morphological and phylogenetic analyses have identified *M. pseudophaseolina* (SARR et al., 2014), *M. euphorbiicola* (MACHADO et al., 2019), *M. vaccinii* (ZHAO et al., 2019), and *M. tecta* (POUDEL et al., 2021). In Brazil, *M. phaseolina*, *M. pseudophaseolina*, and *M. euphorbiicola* have been reported to date.

Macrophomina phaseolina (Tassi) Goid. is a polyphagous pathogen attacking over 500 botanical species (FARR; ROSSMAN, 2025), including major

crops such as beans, soybeans, and melons (NEGREIROS et al., 2019). Weeds can also serve as hosts for this pathogen, which exhibits high genetic diversity among isolates (NEGREIROS et al., 2019). Various techniques have been proposed to study this genetic similarity, including molecular analysis via Polymerase Chain Reaction (PCR) using Random Amplified Polymorphic DNA (RAPD) markers (ŽIVANOV et al., 2019; SAMANTA et al., 2021). Using molecular markers to identify genetic similarity provides more conclusive data regarding the effectiveness of control methods for phytopathogenic diseases (COSTA et al., 2020).

While chemical control remains the most common approach, increasing global concern surrounds the indiscriminate use of synthetic fungicides due to their high toxicity to non-target organisms. Furthermore, studies have highlighted the environmental contamination and adverse effects these chemicals pose to human and animal health (LEITE et al., 2024). Consequently, natural compounds, such as plant essential oils, have emerged as potential components for *M. phaseolina* management. Essential oils from the genus *Cymbopogon*, particularly *C. flexuosus*, have demonstrated significant antifungal activity (GAO et al., 2020; PEREIRA et

al., 2022; PARVEEN et al., 2022; MANJULA; PRATHIBHA; MARHOOB, 2023). These oils may also meet the Organic Materials Review Institute (OMRI) standards for certified crop pest and disease control (OMRI, 2025).

In this sense, this study aimed to evaluate the capacity of RAPD markers to determine the genetic similarity among *M. phaseolina* isolates and to assess the efficacy of *C. flexuosus* essential oil in inhibiting the *in vitro* growth of genetically distinct isolates.

MATERIALS AND METHODS

Macrophomina phaseolina isolates

Thirty *M. phaseolina* isolates were utilized in this study, obtained from the fungal culture collection of Phytopathology Laboratory II at the Universidade Federal Rural do Semi-Árido (Table 1). These isolates had been previously characterized both morphologically and phylogenetically by Negreiros et al. (2019).

Table 1. *Macrophomina phaseolina* isolates used in this study.

ID	Strain number	GenBank access number	Location
1	CMM – 4742	MH373466	Icapuí, CE, Brazil
2	CMM – 4747	MH373437	Icapuí, CE, Brazil
3	CMM – 4737	MH373442	Icapuí, CE, Brazil
4	CMM – 4738	MH373461	Icapuí, CE, Brazil
5	CMM – 4739	MH373457	Icapuí, CE, Brazil
6	CMM – 4740	MH373465	Icapuí, CE, Brazil
7	CMM – 4733	MH373464	Icapuí, CE, Brazil
8	CMM – 4734	MH373440	Icapuí, CE, Brazil
9	CMM – 4735	MH373440	Icapuí, CE, Brazil
10	CMM – 4736	MH373436	Icapuí, CE, Brazil
11	CMM – 4741	MH373443	Icapuí, CE, Brazil
12	CMM – 4743	MH373453	Icapuí, CE, Brazil
13	CMM – 4744	MH373458	Icapuí, CE, Brazil
14	CMM – 4745	MH373467	Icapuí, CE, Brazil
15	CMM – 4746	MH373462	Icapuí, CE, Brazil
16	CMM – 4748	MH373438	Icapuí, CE, Brazil
17	CMM – 4749	MH373444	Mossoró, RN, Brazil
18	CMM – 4750	MH373445	Mossoró, RN, Brazil
19	CMM – 4752	MH373454	Mossoró, RN, Brazil
20	CMM – 4754	MH373447	Mossoró, RN, Brazil
21	CMM – 4751	MH373446	Mossoró, RN, Brazil
22	CMM – 4753	MH373459	Mossoró, RN, Brazil
23	CMM – 4756	MH373460	Mossoró, RN, Brazil
24	CMM – 4758	MH373450	Mossoró, RN, Brazil
25	CMM – 4759	MH373449	Assú, RN, Brazil
26	CMM – 4760	MH373439	Assú, RN, Brazil
27	CMM – 4763	MH373451	Mossoró, RN, Brazil
28	CMM – 4755	MH373463	Mossoró, RN, Brazil
29	CMM – 4762	MH373456	Mossoró, RN, Brazil
30	CMM – 4764	MH373455	Mossoró, RN, Brazil

ID = Identification number of isolates; CE = Ceará; RN = Rio Grande do Norte.

DNA extraction, analysis with specific gene primers and RAPD markers

For fungal multiplication, isolates were cultured on PDA (potato dextrose agar) medium and incubated in a BOD chamber (biochemical oxygen demand incubator) at 30 ± 1 °C in the dark. After five days, fungal colonies were used for genomic DNA extraction with the Macherey-Nagel NucleoSpin™ Microbial DNA Kit, following the manufacturer's instructions. Genomic DNA was quantified by agarose gel electrophoresis (DASH; SHRIVASTAVA, 2020).

Specific gene primers, MpKFI (5'-CCGCCAGAGGACTATCAAAC-3') and MpKRI (5'-CGTCCGAAGCGAGGTGTATT-3') (BABU et al., 2007) were used. Reaction mixtures and amplification conditions followed Silva et al. (2023). Amplified products were separated by electrophoresis at 110 V for 70 min on 1.5% agarose gel prepared with 1× TBE buffer and stained with ethidium bromide ($1 \mu\text{g mL}^{-1}$).

For RAPD-PCR, an initial screening of 100 RAPD primers from the OPA, OPAA, OPD, OPH, and OPM series was performed using DNA from two *M. phaseolina* isolates, 4 (CMM-4738) and 5 (CMM-4739). Amplification reactions were carried out in a final volume of 13 μL containing 10 ng DNA, 1× reaction buffer, 0.25 g mL^{-1} purified BSA, 0.33 μM RAPD primer, 0.16 mM dNTPs, and 1 U μL^{-1} Taq DNA polymerase. Reactions were performed in a TX96 Plus Amplitherm thermocycler programmed for initial denaturation at 94 °C for 1 min, followed by 40 cycles of denaturation at 92 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 5 min. Amplified products were separated under the same electrophoresis conditions described above.

Genetic diversity was assessed based on fragment patterns generated by each primer. A binary matrix was constructed by assigning 1 for presence and 0 for absence of polymorphic bands. Then, a genetic similarity matrix was estimated using Jaccard's coefficient, and cluster analysis was performed by the UPGMA (unweighted pair group method with arithmetic mean). Mean distances were used to determine the degree of similarity among individuals or groups, and the cophenetic correlation coefficient (r) was calculated to assess clustering reliability. Statistical analyses were performed using NTSYS-pc 2.1.

Effect of *Cymbopogon flexuosus* essential oil on the growth of *Macrophomina phaseolina*

The experiment was conducted with *M. phaseolina* isolates 5 (CMM-4739) and 29 (CMM-4762). These isolates were selected because they differ in disease severity, exhibiting low and high aggressiveness in melon plants, respectively (NEGREIROS et al., 2019; SALES JÚNIOR et al., 2020). *Cymbopogon flexuosus* essential oil from Terraflor was used.

Treatments consisted of the two isolates, six oil concentrations (0.02, 0.05, 0.10, 0.15, 0.20, and 0.25%), a negative control (PDA medium), and positive control (PDA

medium supplemented with Maxim fungicide). The experiment followed a completely randomized design with eight replicates per treatment.

PDA medium was autoclaved for 15 min at 121 °C and 1 atm. After cooling to approximately 45 °C, essential oil concentrations were added to the medium using an automatic pipette. Under aseptic conditions, the medium was then poured into 90 × 90 mm Petri plates. After solidification, 8 mm mycelial discs were removed from actively growing fungal cultures and transferred to the center of each plate. Plates were incubated in a BOD chamber at 30 ± 2 °C.

Mycelial growth was evaluated by measuring colony diameter in two perpendicular directions when fungal growth in the control treatment reached the edge of the dish, which occurred after three days. Percentage inhibition of growth (% PIG) was calculated as: $\% \text{PIG} = (\text{growth in control plate} - \text{growth in treated plate}) / \text{growth in control plate} \times 100$.

A four-parameter logistic model was used to predict the effect of essential oil on *M. phaseolina* mycelial growth using *TableCurve*. Means between isolates were compared using the Mann-Whitney test at the 5% probability level.

RESULTS AND DISCUSSION

DNA extraction, analysis with specific gene primers, and RAPD markers

PCR product fragments of approximately 320 bp were detected in all thirty *M. phaseolina* isolates, as expected according to Babu et al (2007). Of the 100 RAPD primers evaluated, 13 showed greater polymorphism and were therefore selected for assessment of genetic diversity (Table 2). These 13 RAPD primers amplified 135 loci, of which 97 (71.85%) were classified as polymorphic.

The average number of polymorphic bands per primer was 7.5, which was considered satisfactory for detecting polymorphism with the selected primers. Moreover, PIC values ranged from 0.24 (OPM 13) to 0.46 (OPAA 17). An average of five polymorphic bands per primer has been considered adequate for polymorphism detection (MAZUMDAR et al., 2020).

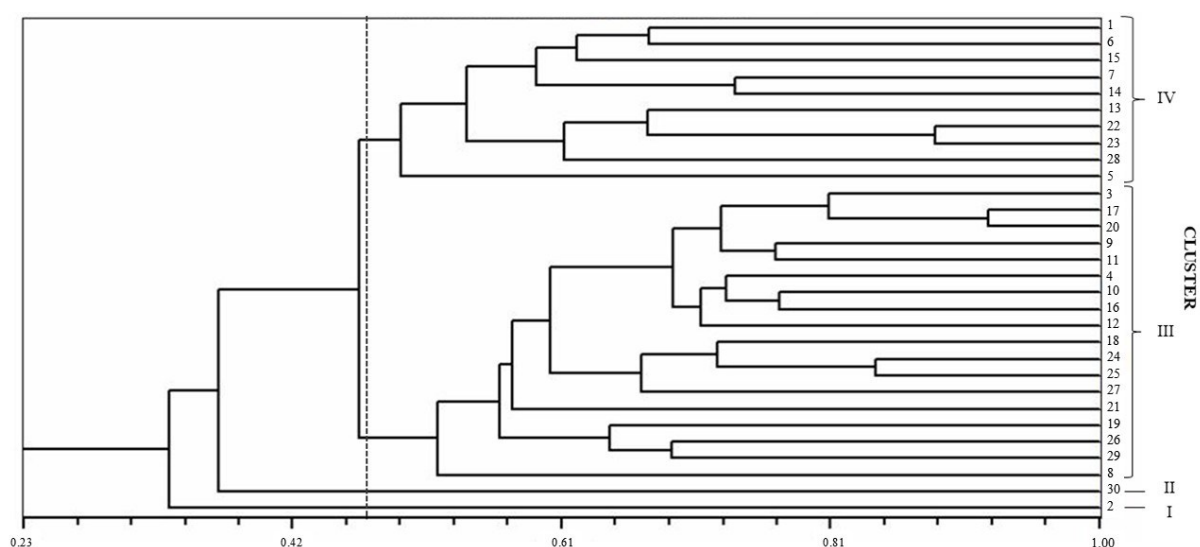
High PIC values and substantial variation were also observed, consistent with the findings of Mahdizadeh, Safaie, and Goltapeh (2011). This variation may be associated with differences in primer sets used among studies. The primers selected in the present study revealed high genetic variability and were therefore suitable for evaluating genetic diversity among *M. phaseolina* isolates.

A dendrogram was generated using the UPGMA hierarchical method, and a cutoff value of 47% genetic similarity separated the isolates into four groups. Groups III and IV were further divided into subgroups (Figure 1). Grouping in the dendrogram was not associated with host species or geographical origin of the isolates. Isolates 2 (CMM-4747) and 7 (CMM-4733) were the most genetically distant, showing the lowest similarity coefficient (0.23), whereas isolates 17 (CMM-4749) and 20 (CMM-4754) showed the highest genetic similarity (0.92).

Table 2. RAPD primers used for DNA amplification of *Macrophomina phaseolina* genotypes and corresponding results.

Primer	Sequence	TL	ML	PL	%P	PIC
OPAA 4	5'- AGGACTGCTC - 3'	8	2	6	75%	0.30
OPAA 6	5'- GTGGGTGCCA - 3'	4	1	3	75%	0.36
OPA A 10	5'- TGGTCGGGTG - 3'	13	4	9	69%	0.34
OPAA 14	5'- AACGGGCCAA - 3'	13	3	10	77%	0.27
OPAA 15	5'- ACGGAAGCCC - 3'	14	4	10	71%	0.34
OPAA 17	5'- GAGCCCGACT - 3'	12	6	6	50%	0.46
OPAA 18	5'- TGGTCCAGCC - 3'	7	1	6	86%	0.39
OPH 2	5'- TCGGACGTGA - 3'	11	1	10	91%	0.42
OPH 4	5'- GGAAGTCGCC - 3'	9	4	5	56%	0.39
OPM 2	5'- ACAACGCCTC - 3'	12	3	9	75%	0.32
OPM 3	5'- GGGGGATGAG - 3'	12	4	8	67%	0.28
OPM 5	5'- GGGAACGTGT - 3'	7	1	6	86%	0.34
OPM 13	5'- GGTGGTCAAG - 3'	13	4	9	69%	0.24
Total	-	135	38	97	-	
Mean	-	10.4	2.9	7.5	-	0.34

TL: total *loci*; ML: monomorphic *loci*; PL: polymorphic *loci*; %P: polymorphism percentage; PIC: polymorphism information content.


Figure 1. Cluster analysis of 30 *Macrophomina phaseolina* isolates based on the genetic distance matrix calculated using Jaccard's similarity coefficient.

RAPD markers are arbitrarily amplified, highly polymorphic dominant markers. They require moderate amounts of DNA and involve intermediate technical and operational costs. Because RAPD uses random primers, polymorphisms can be analyzed without prior knowledge of the target organism's DNA sequence, which is one of the main advantages of this technique (OLIVEIRA; AZEVEDO, 2022). However, a major limitation is the low reproducibility of results and, despite this limitation, RAPD markers remain widely used in studies of fungal population genetics (ŽIVANOV et al., 2019; SAMANTA et al., 2021).

Moreover, Živanov et al. (2019) also reported similar

findings when investigating genetic variability in *M. phaseolina* isolates collected from six countries (Turkey, Bulgaria, Romania, Spain, Ukraine, and Serbia) and from different hosts, including sunflower (*Helianthus annuus*), maize (*Zea mays*), soybean (*Glycine max*), flax (*Linum usitatissimum*), common bean (*Phaseolus vulgaris*), and zebra plant (*Aphelandra squarrosa*).

The genetic variability observed among the isolates in the present study may be associated with differences in pathogenic severity. The most divergent isolates, 2 (CMM-4747) and 7 (CMM-4733), showed contrasting severity scores in a pathogenicity test conducted by Sales Júnior et al. (2020) on cowpea.

The degree of genetic similarity observed in the present study was greater than that reported by Martínez-Hilders and Laurentin (2012), who evaluated phenotypic and molecular characteristics of *M. phaseolina* isolates from the sesame-producing region of Venezuela. In the present study, analysis of thirty isolates allowed estimation of a cophenetic correlation coefficient of $r = 0.84$, indicating good agreement between the dendrogram and the similarity matrix generated using Jaccard's coefficient. These results support the reliability of RAPD markers for assessing genetic diversity in *M. phaseolina*.

Effect of *Cymbopogon flexuosus* essential oil on the growth of *Macrophomina phaseolina*

In the assay evaluating the effect of *Cymbopogon flexuosus* essential oil on the growth of *M. phaseolina* isolates, all tested concentrations inhibited mycelial growth of isolates 5 (CMM-4739) and 29 (CMM-4762). Percentage inhibition of growth ranged from 2 to 100% (Figure 2). In addition, logistic regression analysis showed that percentage inhibition of growth (%PIG) increased with increasing essential oil concentration.

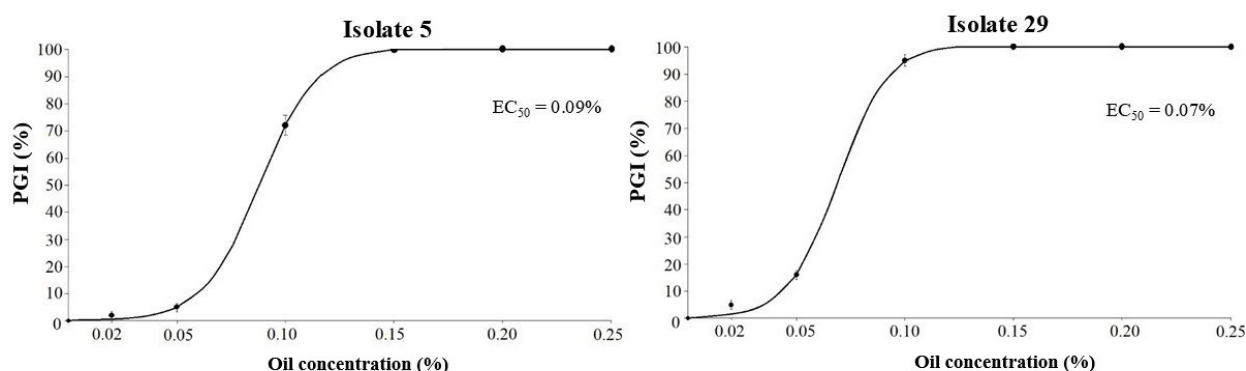


Figure 2. Logistic regression curves and half-maximal effective concentration (EC_{50}) for each *Macrophomina phaseolina* isolate exposed to *Cymbopogon flexuosus* oil. EC_{50} represents the concentration required to cause 50% inhibition of mycelial growth, as estimated from the regression equation.

According to EC_{50} analysis, the essential oil concentration required to induce 50% growth inhibition in isolate 5 (CMM-4739) was 0.09%. For isolate 29 (CMM-4762), a lower concentration of 0.07% was required (Figure 2). Maximum percentage inhibition of growth (PIG = 100%) was achieved at an essential oil concentration of 0.15% for both *M. phaseolina* isolates. Therefore, this concentration was considered the minimum required for complete inhibition of mycelial growth in the evaluated isolates.

Regarding percentage inhibition of growth between isolates 5 (CMM-4739) and 29 (CMM-4762), no significant difference in inhibitory effect was detected between the two *M. phaseolina* isolates according to the Mann-Whitney test ($p > 0.05$) (Figure 3). These results indicate that *C. flexuosus* essential oil was effective in suppressing *M. phaseolina* mycelial growth, including two genetically divergent isolates.

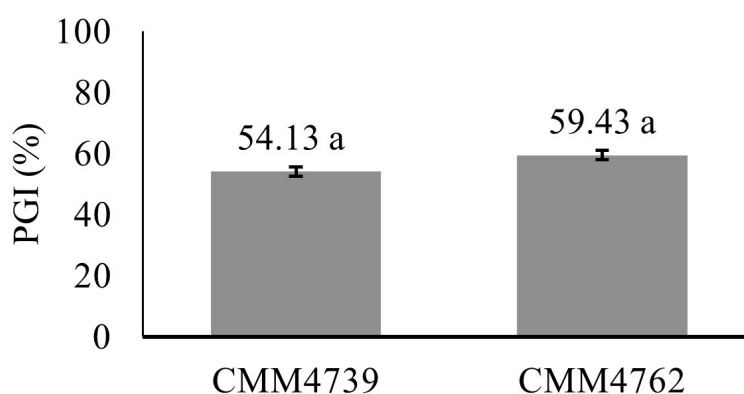


Figure 3. Percent growth inhibition (PGI) of *Macrophomina phaseolina* isolates treated with *Cymbopogon flexuosus* essential oil.

Regarding the effect of *C. flexuosus* essential oil, percentage inhibition of *M. phaseolina* growth increased as oil concentration increased. Similarly, Costa, Mora and Millezi (2019) reported that *C. flexuosus* essential oil contains biologically active compounds, with geranial and neral isomers as major constituents.

Likewise, Parikh, Agindotan, and Burrows (2021),

while evaluating the antifungal activity of plant-derived essential oils against pulse crop pathogens, found that *C. flexuosus* essential oil completely inhibited mycelial growth of *Aphanomyces* and *Pythium*, and also reduced growth of *Botrytis*, *Colletotrichum*, *Didymella*, *Fusarium*, *Stemphylium*, and *Sclerotium*.

The present results showed that *C. flexuosus* essential

oil was effective for *in vitro* inhibition of *M. phaseolina* growth at lower concentrations than those reported for *C. citratus* essential oil. Pandey (2003) found that *C. citratus* oil inhibited growth of *Colletotrichum gloeosporioides* at concentrations of 1.0 and 1.5%. This difference may be attributed to variation among pathogen species, as well as differences in chemical composition between essential oils extracted from distinct plant species within the genus *Cymbopogon*.

Lemongrass oil contains citral, nerol, geraniol, and linalool, compounds known for strong antifungal activity (SARKAR et al., 2024). The antifungal action of citral may be associated with its ability to form charge-transfer complexes with electron donors in fungal cells. In addition, the hydrophobic nature of essential oils allows them to partition into lipids of fungal cell membranes and mitochondria, disrupting membrane structure, increasing permeability, and causing leakage of ions and other cellular constituents (KUMAR et al., 2008).

Cymbopogon flexuosus essential oil inhibited mycelial growth of the *M. phaseolina* isolates evaluated in this study, demonstrating consistent antifungal activity against the pathogen.

CONCLUSION

The RAPD marker was effective for assessing genetic diversity among *Macrophomina phaseolina* isolates. *Cymbopogon flexuosus* essential oil showed strong inhibitory potential against *M. phaseolina*, reducing mycelial growth of the isolates at most concentrations assessed under *in vitro* conditions. This genetic diversity should be considered when designing disease management strategies, including germplasm screening assays as well as *in vitro* and *in vivo* evaluations.

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