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CROP PROTECTION

Preservation of moderately resistant or tolerant genotypes: a strategy to overcome guava decline

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ABSTRACT: Native to the tropical Americas, guava (*Psidium guajava* L.*)* is an important crop in Brazil. However, the emergence of so-called guava decline, a complex disease resulting from root parasitism by the root-knot nematode (*Meloidogyne enterolobii* Yang & Eisenback) in association with opportunistic fungi, has decimated guava orchards across Brazil and in other countries. In the present study, seminiferous guava accessions were vegetatively propagated by minigrafting and their genotypes preserved for resistance reassessment in clones to confirm or not host plant reactions. The results indicated a highly virulent parasite, high host suitability of the *P. guajava* species, and widely varying reactions among plants of the same genotype and between different genotypes, demonstrating that the strategy of preserving the germplasm and reassessing reactions in clones may be important in identifying and selecting germplasms with a degree of resistance to *M. enterolobii*. The progeny of cv. Paluma P02R5R2 obtained the lowest average parasite reproduction factor (RF = 22.11) among the genotypes evaluated and was; therefore; classified as moderately resistant and preserved for future research.
Key words: genetic variability, cv. Paluma, reproduction factor, minigrafting, *Meloidogyne enterolobii*.

Preservação de genótipos moderadamente resistentes ou tolerantes: uma estratégia para suplantar o declínio da goiabeira

RESUMO: Originária da América Tropical, a goiabeira (*Psidium guajava* L.*)* tem grande relevância para o Brasil. Contudo, o surgimento do patossistema designado como declínio da goiabeira, problema fitossanitário provocado pelo parasitismo das raízes pelo nematoide-das-galhas (*Meloidogyne enterolobii* Yang & Eisenback) em associação com fungos oportunistas, dizimou muitos pomares em todas as regiões do Brasil e em outros países. No presente estudo, propagou-se vegetativamente, por miniestaquia, acessos de goiabeiras seminíferas mantendo seus genótipos preservados e reavaliando a resistência por meio dos clones de forma a comprovar ou não as reações das plantas hospedeiras. Os resultados apontam para alta virulência do parasita, bem como alta hospedabilidade da espécie *P. guajava*, além da existência de grande variação da reação entre plantas do mesmo genótipo e entre genótipos distintos, o que indica que a estratégia de preservação do germoplasma e a reavaliação da reação em clones pode ser importante na busca e seleção de germoplasma com algum grau de resistência a *M. enterolobii*. A progênie da cv. Paluma P02R5R2 obteve a menor média de Fator de Reprodução do parasita (FR = 22,11) entre os genótipos avaliados, sendo classificada como moderadamente resistente e preservada para estudos posteriores.
Palavras-chave: variabilidade genética, cv. Paluma, fator de reprodução, miniestaquia, *Meloidogyne enterolobii*.

INTRODUCTION

Data from the 2020 Municipal Agricultural Production survey (IBGE, 2021) indicate that Northeastern Brazil is the leading producer of guava in the country. Despite of the damage caused by *Meloidogyne enterolobii*, guava is among the most widely grown fruit trees in the region, covering 10,605 ha and accounting for 48.15% of the total cultivated area.

The parasite was first identified in Brazilian commercial guava orchards in 2001, in the municipalities of Petrolina in Pernambuco state (PE) and Juazeiro in Bahia state (BA) (CARNEIRO et al., 2001). Once the nematode is detected, the useful life of the orchard declines drastically (SILVA et al., 2014), leading to plant death as a function of greater or lesser soil infestation (CASTRO, 2019).

Initially, *M. enterolobii* was considered solely responsible for the decline of infected guava trees, but GOMES et al. (2011) subsequently proved interaction with *Fusarium solani* (Mart.) Sacc. in root deterioration. Since then, guava decline has been treated as a complex disease resulting from the combined action of these two pathogens. Recent studies classified the etiology of the causal agent associated with guava decline to the fungus *Neocosmosporafalciformis* (Carrión) L. Lombard & Crousas opposed to *F. solani* (VELOSO et al., 2020).

In 2019, Embrapa Semiárido launched the resistant rootstock BRS Guaraçá, a hybrid cultivar

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resulting from a single cross between common guava accession Gua161PE (*P. guajava* L.) and Brazilian guava accession Ara138RR (*Psidium guineense* Sw.) (CASTRO, 2019). BRS Guaraçá is currently the only resistant rootstock compatible with commercial guava varieties capable of withstanding nematode infection.

Research continues to focus on the search for nematode-resistant rootstocks, since this alternative prevents the parasite from damaging the roots, which are the gateway for fungal infection (CASTRO, 2019). Prospection studies are ongoing in both guava and other crops considered susceptible to *M. enterolobii*, with promising results. The individuals studied showed reproduction factor (RF) variability, with the lowest values indicating resistance to the pathogen (COSTA FILHO et al., 2018; MIRANDA et al., 2012; OLIVEIRA et al., 2019).

Determining RF in plant pathogen experiments is a destructive process, even when a genotype is found to be a source of resistance, its progenies are not available for reassessment to prove its effectiveness against the nematode and for subsequent use as rootstock for commercial guava trees (OLIVEIRA et al., 2019).Thus, methods aimed to propagate and maintain plants from resistant/tolerant progenies are needed in order to preserve the shoots.

Minigrafting is a technique capable of contributing to plant breeding research, since it involves exploiting the juvenile and hormonal potential of shoots obtained from seedlings produced by seeds or cuttings, in order to induce rooting and rapid formation of vigorous clones (ALFENAS et al., 2004; FERRIANI et al., 2010).This technique can be used to propagate guava trees and is considered beneficial for breeding programs aimed at selecting pest and disease-resistant genotypes (MARINHO et al., 2009).

The present study aimed to propagate seminiferous guava accessions via minigrafting for *Meloidogyne* resistance assessment, preserve the genotypes and reassess clonal tolerance to prove or not the previously observed reactions. This technique could be an important tool in maintaining guava genetic resources with the potential to overcome guava decline for subsequent use of these genotypes in breeding research to select resistant rootstocks or plants with desirable commercial traits.

MATERIALS AND METHODS

The study was conducted between March 2021 and January 2022, in a greenhouse belonging to the Department of Technology and Social Sciences of the State University of Bahia (UNEB), Campus III, in the municipality of Juazeiro - BA (9°25'10.67"S, 40°29'8.24"W, altitude of 368m), located in the driest region of the country. Climate in the region is classified as BShor semiarid according to Köppen's classification, with average annual temperature and rainfall of >18°C and <800mm, respectively (ALVARES et al., 2013).

The seminiferous material used to produce the mother plants belongs to the *Psidium* spp. germplasm of UNEB, kept in cold storage (10 ºC) and 40% relative humidity at Embrapa Semiárido.

Mother plants with very low RF values (1.2 to 2.69) and a cv. Paluma plant considered a susceptibility standard (RF = 231.75) were selected and propagated by minigrafting to create a mini-clonal garden (Figure 1). Propagation was carried out in a greenhouse covered in black 50% shade cloth, with intermittent spray irrigation every three minutes, lasting 10 seconds per application.

The eight accessions selected (Figure 1) produced 15 seedlings per minigraft, since some mother plants provided more than one minicutting, all the resulting seedlings were preserved. Thus; although, some treatments were evaluated separately as a different source of variation, they originated from the same mother plant.

At 125 days after propagation (DAP) by minigrafting, the seedlings were transplanted to 12-liter pots and the resulting shoots subsequently extracted for further multiplication by serial minigrafting in a mist chamber. Each shoot provided at least six minicuttings of the subculture, producing a new batch of seedlings (Figure 1) for inoculation with *M. enterolobii* and assessment of whether or not the RF was preserved in the selected genotypes.

Seventy days after the onset of the second propagation cycle, the young seedlings were transferred to plastic bags suitable for seedlings, containing 1.05 kg of commercial substrate at the bottom of the bag, covered with 4.2 kg of autoclaved sandy soil, up to approximately 5 cm from the top of each recipient.

To obtain the *M. enterolobii* inoculum, the roots of parasitized guava trees were collected from an area containing plants that remained after the eradication of an orchard located in Projeto Salitre, Juazeiro (BA) (9°32'16.74"S, 40°37'15.03"W, altitude of 379.43m). Species identification was confirmed based on detection of the esterase phenotype En4 (Rm: 0.73; 0.80; 0.90; 0.97), characteristic of *M. enterolobii* (ALFENAS et al., 1991; SANTOS et al., 2020).

The material collected was processed to extract the eggs and second-stage juveniles (J2) with

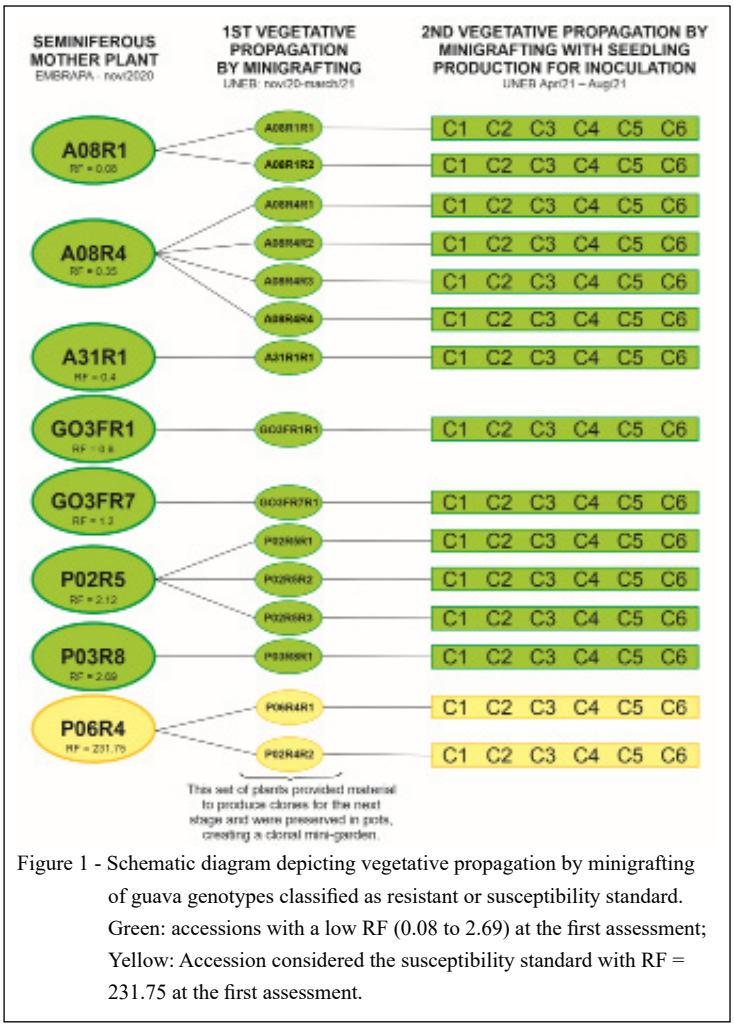

Figure 1 - Schematic diagram depicting vegetative propagation by minigrafting of guava genotypes classified as resistant or susceptibility standard. Green: accessions with a low RF (0.08 to 2.69) at the first assessment; Yellow: Accession considered the susceptibility standard with RF = 231.75 at the first assessment.

a 0.5% sodium hypochlorite, using a blender instead of manual agitation, followed by centrifugation and flotation, according to the combined methods of BONETI & FERRAZ (1981) and COOLEN & D'HERDE (1972) described by MACHADO et al. (2019).

The inoculum suspension was submitted to counting and calibration using an optical microscope and counting chamber, adjusting the approximate concentration to 600 eggs + J2 per mL. Next, the seedlings were inoculated with 3 mL of the suspension in 1 mL aliquots, applied to three small holes near the base of the plant using a graduated pipette.

At 135 days after inoculation (DAI), the best time to evaluate infection according to BURLA et al. (2010), destructive assessment of the root system was performed in the laboratory to determine the following variables: shoot height (SH), longest root length (LRL), shoot fresh weight (SFW), root fresh weight (RFW), total plant fresh weight (TPFW), shoot to root ratio (S:R –ratio between SFW and RFW), gall index (GI), final population (FP), final population per root gram (FP/Rg) and reproduction factor (RF).

Once again, the extraction methods of BONETI & FERRAZ (1981) and COOLEN & D'HERDE (1972) were applied, whereby the suspension containing eggs + J2 corresponding to each plant was stored in a labelled individual collector, followed by an approximate count of the final population consisting of eggs + J2 present in the root system of each experimental unit.

The gall index (GI) was determined based on a score from 0 to 5, where 0: no galls, 1: 1-2 galls, 2: 3-10 galls, 3: 11-30 galls, 4: 31-100 galls, 5: > 100 galls per root system (TAYLOR & SASSER, 1978). The reproduction factor was calculated by the formula RF = final population (FP) / initial population (IP) (OOSTENBRINK, 1966).

Resistance was classified according to the system proposed by MOURA & RÉGIS (1987), considering the reproduction factor reduction (RFR)

per treatment, expressed in percentage, whereby RFR = 0 to 25%: highly susceptible (AS); 26 to 50%: susceptible (S); 51 to 75%: low resistance (LR); 76 to 95%: moderately resistant (MR); 96 to 99% resistant (R); and 100%: highly resistant (HR) or immune (I).

The experimental design was completely randomized (CRD), with 15 treatments (plants from the 1st propagation) and six repetitions (seedlings from the 2nd propagation).For statistical analyses, the data for the variables SFW, RFW, TPFW, R:S, FP and RF were transformed by extracting $\sqrt{x}$, and $\sqrt[4]{x}$ was extracted for FP/Rg to meet assumptions of normality, homoscedasticity and homogeneity, followed by analysis of variance (ANOVA). SISVAR software was used for the analyses and means were grouped by the Scott - Knott test at 5% ($P < 0.05$).

The plants in the clonal mini-garden have been maintained for use as propagative material in future research.

RESULTS AND DISCUSSION

The plants in the clonal mini-garden were successfully propagated using minicuttings (second-generation clones).

Under intermittent mist irrigation, the propagules achieved 100% establishment and the onset of rooting was observed at 23 DAP.

From 105 DAI onwards, some plants showed symptoms of chlorosis, leaf loss,as well as dieback and wilting of young leaves at the hottest time of day. When roots emerged through different points in the plastic container, from the 18th week of parasitism, small discreet galls were visible, indicating inoculum viability and that the nematodes had caused hyperplasia in root cells.

Assessment at 135 DAI demonstrated no statistically significant variations in the morphometric data LRL, SFW, RFW and TPFW. However, there were significant differences in SH and R:S, since the Scott-Knott test at 5% separated the genotypes into two subgroups for both these variables (Table 1).

SH values in the group with the tallest plants varied between 96.90 and 120.23 cm, with values of 70.83 to 93.55 cm among the smallest plants. Treatment A08R4R4 obtained the highest average in the first group (120.23 cm). For R:S, despite the separation into two subgroups, only three treatments stood out for this variable (G03FR1R1, A08R4R4 and P06R4R2), with the best result recorded for GO3FR1R1 with an average of 0.94, that is, the root and shoot biomass of the plants were similar.

This raises the hypothesis that these accessions are better able to regulate water absorption and photosynthetic activity, a positive and desirable trait in a genotype that responds to stress and biotic root damage with increased shoot and root emission to compensate for losses.

None of the *cv.* Paluma treatments assessed obtained RF values that confirmed resistance (RF < 1) or immunity (RF = 0) according to the standard classification (OOSTENBRINK, 1966), corroborating the results of other studies that identified *Psidium guajava* as susceptible to *M. enterolobii* (BIAZATTI et al., 2016; CARNEIRO et al., 2012; CASTRO et al., 2012; MIRANDA et al., 2012).Additionally, in the native guava accessions studied here, in both cases an important variation was observed in parasite RF, demonstrating moderate resistance. As such, the classification adapted by MOURA & RÉGIS (1987) was adopted to rank host suitability (Table 2).

The lowest average FP (39.812) and RF (22.11) were recorded for a *cv.* Paluma progeny (P02R5R2), with statistically significant differences ($P < 0.05$) detected by ANOVA. This progeny stood out from the others in the third group of means, according to the Scott-Knott test (Table 2). Differences were observed between treatments and within groups of clones from the same ministration and those from the same seminiferous mother plant, that is, from the same genotype. This is relevant because despite the consensus that the same vegetatively propagated genotype produces offspring identical to itself, studies such as that of DALAGNOL (2010) indicated that conventional vegetative propagation, used in our study, results in epigenetic variations that alter the phenotype due to DNA methylation. Careful analysis of table 2 shows that two to four minicuttings were used for conventional vegetative propagation of four accessions (A08R1, A08R4, P02R5 and P06R4), whose reaction to *M. enterolobii* was evaluated. Among plants from a same group of mini-cuttings removed from a same plant, means were observed in different groups for FP, FP/Rg and RF in accessions A08R1, P02R5 and P06R4, according to the Scott-Knott test ($P < 0.05$). Although, no significant differences were recorded for these three variables in A08R4, RFR increased from 0.00 to 43.21%, and when the dataset for this trait was considered in all four accessions, the variation observed was from 0.00 to 93.42% (Table 2).The data clearly demonstrated epigenetic variation in the expression of all four variables.

These findings are similar to those reported by MIRANDA et al. (2010), who studied selection methods for *M. enterolobii*-resistant *Psidium* spp.

Table 1 - Means of the biometric parameters measured at 135 days after inoculation.

Treatment	-------SH-------	--------LRL-----	------SFW------	------RFW------	-----TPFW-----	-------R:S-------
A08R1R1	116.03 a	49.83	182.06	112.36	294.42	0.63 b
A08R1R2	109.77 a	53.77	202.36	106.47	308.82	0.52 b
A08R4R1	100.32 a	57.05	145.66	98.48	244.14	0.68 b
A08R4R2	87.38 b	52.32	160.55	105.18	265.72	0.64 b
A08R4R3	96.90 a	45.67	169.76	101.12	270.88	0.61 b
A08R4R4	120.23 a	57.00	178.74	152.00	330.74	0.86 a
A31R1R1	70.83 b	52.25	86.25	59.33	145.58	0.71 b
GO3FR1R1	85.60 b	49.07	139.25	125.18	264.43	0.94 a
GO3FR7R1	79.38 b	53.23	138.50	109.69	248.20	0.72 b
P02R5R1	85.17 b	50.92	134.17	96.07	230.24	0.68 b
P02R5R2	72.65 b	44.32	107.86	58.31	166.17	0.49 b
P02R5R3	105.20 a	51.00	153.71	99.47	253.18	0.66 b
P03R8R1	93.55 b	51.97	129.73	71.27	201.00	0.53 b
P06R4R1	116.45 a	55.02	141.20	82.05	223.24	0.58 b
P06R4R2	113.62 a	47.90	148.07	121.77	269.84	0.79 a
CV (%)	23.61	14.49	20.54	27.50	22.35	15.08
Fcal	3.08^{*}	1.47^{NS}	1.55^{NS}	1.46^{NS}	1.41^{NS}	2.51^{*}

Means of six repetitions per treatment. Variables:shoot height in cm (APA), longest root length in cm (LRL), shoot fresh weight in g (SFW), root fresh weight in (RFW), total plant fresh weight in g (TPFW) and ratio between root and shoot fresh weight (R:S).
Values followed by the same lowercase letter in the column indicate a group of means that do not differ according to the Scott-Knott test at 5% significance.
*significant at 1%, ^{NS} (no significant).

genotypes and obtained coefficients of variation of 25 to 171% between vegetatively propagated plants of the same accession, including wild guava individuals with low RF values (0.4 – 2.6).

The seminiferous mother plants of the genotypes reassessed in the present study, propagated by minigrafting, were classified as resistant or as exhibiting low nematode production (RF of 0.08 to 2.69) in a previous investigation. It can; therefore, be hypothesized that the inoculums of the populations collected in different locations (Casa Nova – BA and Juazeiro – BA) varied in terms of physiology or the mode of action of the parasites; however, pathogenicity tests were not conducted to confirm this hypothesis.

Another relevant factor to consider is that the seminiferous mothers had underdeveloped root systems with low fresh weight at the time of assessment, which significantly limits parasite infection and multiplication. However, the clones obtained by vegetative propagation produced an abundant network of tendrils, which facilitated infection and enhanced nematode reproduction. Nevertheless, materials whose average RF was considered high showed a significant decline in RF in relation to the susceptibility standard. In the present study, RF declined by 75.09 to 93.42% (Table 2) and as such, these moderately resistant progenies should not be disregarded.

Similar results, requiring further research, were obtained by FREITAS et al. (2014), who assessed the resistance of *Psidium* spp. accessions from Embrapa Recursos Genéticos e Biotecnologia (formerly CENARGEN) to root-knot nematodes. According to the authors, of the 44 *P. guajava* accessions tested, only one (wild guava) obtained an RF of 22.9, while the highest value recorded among the accessions was 943.4.

Interestingly, in our study, the reaction to infection in both groups of plants from progeny P06R4, previously classified as the susceptibility standard (FR = 231,75), reduced FP in second-generation clones by 41.89 and 73.37% on average (Table 2).

CAVALCANTI JUNIOR et al. (2020) studied guava accessions and obtained RFs of 1.43 (HU-RJ-G01) and 1.69 (PAU-CM-G03), indicating variability in their reactions to nematodes, showing potential as sources of resistance. As such, there is a need for research on interaction between different inoculum sources and moderately resistant guava genotypes that considers aspects of qualitative and quantitative resistance, in order to determine which factors explain the superior resistance of this plant

Table 2 - Reaction to *M. enterolobii* in guava genotypes (*P. guajava* L.) propagated by minigrafting, assessed at 135 DAI with 1,800 eggs + J2/plant in a greenhouse, where RF = FP/1800.

Treatment	RFW[1]	GI	FP[1, 2]	FP/gR[1, 2]	RF[1, 2]	RFR[3]	C[4]
A08R4R2*	105.18	4.83	604,492 a	6,991.04 a	335.82 a	00.00	AS
P03R8R1	71.27	5	522,468 a	8,481.66 a	290.26 a	13.57	AS
A08R4R4	152.00	5	515,257 a	3,523.82 a	286.25 a	14.76	AS
A08R1R1	112.36	4.83	486,273 a	4,919.52 a	270.15 a	19.56	AS
P02R5R3	99.47	5	433,038 a	4,796.53 a	240.57 a	28.36	S
A08R4R1	98.48	5	408,707 a	4,153.68 a	227.06 a	32.39	S
A31R1R1	59.33	5	398,448 a	7,074.92 a	221.36 a	34.08	S
P06R4R1	82.05	5	351,278 a	4,870.44 a	195.15 a	41.89	S
A08R4R3	101.12	4.83	343,275 a	5,457.88 a	190.70 a	43.21	S
A08R1R2	106.47	5	215,928 b	2,054.19 b	119.96 b	64.28	PR
P06R4R2	121.77	5	160,992 b	1,741.41 b	89.44 b	73.37	PR
P02R5R1	96.07	5	150,597 b	1,614.14 b	83.66 b	75.09	MR
GO3FR1R1	141.85	4.83	144,975 b	1,928.66 b	80.54 b	76.02	MR
GO3FR7R1	109.69	5	136,242 b	1,922.04 b	75.69 b	77.46	MR
P02R5R2**	58.31	5	39,812 c	789.04 b	22.11 c	93.42	MR
CV (%)			25.63	16.67	25.63		
Fcal			9.06	6.23	9.06		

[1]Means of six repetitions (original biological data).RFW = root fresh weight; GI = gall index (IG), FP = final population, FP/Rg = final population per root gram (FP/RFW), RF = reproduction factor (FP/IP) - where IP = initial population composed of 1,800 eggs and J2, RFR = reproduction factor reduction in relation to the susceptibility standard expressed in %, C = classification, CV = coefficient of variation, and Fcal = calculated F value.

[2]Values followed by the same lowercase letter in the column indicate a group of means that do not differ according to the Scott-Knott test at 5% significance.

[3]Calculated by the formula RFR = [(RF of the susceptibility standard – RF of the treatment) / RF of the susceptibility standard] x 100.

[4]Classification: 0 a 25% - highly susceptible (HS); = 26 to 50% - susceptible (S); = 51 to 75% - low resistance (LR); = 76 to 95% - moderately resistant (MR); = 96 to 99% - resistant (R) and = 100% - highly resistant (HR) or immune (I) (MOURA & RÉGIS, 1987).

material. A plausible approach is to preserve plants with a low RF for more in-depth research to identify the factors that govern interactions between *M. enterolobii* and host guava plants; thereby, enabling the development of tolerant or less susceptible rootstocks or cultivars to provide producers with different options.

Preserving the mother plant in pots filled with sterilized substrate while its offspring, multiplied by minigrafting, is inoculated with the parasite for resistance assessment, as proposed here, is a feasible alternative for use in research with guava genotypes. Identifying resistant guava accessions capable of transmitting this genetic trait remains a desirable goal. A discovery of this magnitude could resolve the issue of compatibility between plants of different species of the genus *Psidium* or interspecific hybrids with commercial varieties, with minigrafting as an ally in both breeding-related research and multiplication of the plant material, in order to make this technology available to farmers and promote the expansion of the current planted area.

CONCLUSION

The progeny of Paluma P02R5R2 showed important resistance in relation to the remaining treatments with the same cultivar (RF = 22.11, that is, 1312% lower than the highest RF of 290.26 recorded for P03R8R1), revealing unique phenotypic potential as a cultivar of commercial *P. guajava*, known to be susceptible, but compatible as rootstock.

The different reactions of the same genotypes inoculated with nematodes from different sources suggest the need for additional research on variability in the mode of action of the parasite. Moreover, differences observed in minicuttings from a same mother plant indicate epigenetic variation in the expression of the variables analyzed, including the reproduction factor.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

The article was taken from the master's dissertation of the first author, with the second author serving as an advisor. All the authors contributed to the conception of the study and the field and laboratory activities, critically reviewed the manuscript, and approved the final version.

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