

Simultaneous cloning and selection of *Psidium* genotypes resistant to *Meloidogyne enterolobii*

Clonagem e seleção simultâneas de genótipos de *Psidium* resistentes a *Meloidogyne enterolobii*

Sydney Pereira Galvão^{1*}; Cláudia Sales Marinho²; Raudielle Ferreira dos Santos³; José Olívio Lopes Vieira Júnior³; Alexandre Pio Viana²; Ricardo Moreira Souza²

Highlights

Nematode resistance varies in *Psidium* and is not uniformly inherited.

Prior minicutting enabled rapid *Psidium* reproduction with proven resistance.

Early cloning preserves resistance to nematodes, simplifying genotype multiplication.

Abstract

One of the obstacles to arboreal plant breeding is the time required between the selection of superior genotypes and their multiplication. This study investigates *Psidium* (guava) hybrids developed to obtain rootstock or new scions resistant to the nematode *Meloidogyne enterolobii*. The use of half-siblings or hybrid seeds of these genetic materials does not preserve the genetic profile of resistant individuals, making destructive selection methods unfeasible. Propagating juvenile *Psidium* material by minicutting produces a high rooting percentage, facilitating the cloning of segregating families and reducing the time required to produce replicas. In this study, segregating families for resistance to *M. enterolobii* were cloned by minicutting, with replicas maintained in clonal minigardens while the mother plants were inoculated and evaluated for nematode reproduction in the root system. The results indicate resistance segregation both among and within families. Early cloning by minicutting demonstrated 100% efficiency, allowing the identification of 30 resistant individuals to occur simultaneously with the first multiplication cycle of these individuals, reducing the time and uncertainty involved in recovering superior materials. The methodology adopted is an effective strategy, allowing advances in guava breeding programs. Additionally, individuals resistant to *M. enterolobii* were observed in the hybrids *P. guajava* x *P. cattleianum*; *P. cattleianum* x *P. guineense* and *P. guineense* x *P. cattleianum*.

Key words: Guava breeding. Minicutting. *Psidium cattleianum*. *Psidium guajava*. *Psidium guineense*.

¹ Doctoral Student in the Graduate Plant Production Program, Universidade Estadual do Norte fluminense Darcy Ribeiro, UENF, Campos dos Goytacazes, RJ, Brazil. E-mail: sydneygalvaoagro@gmail.com

² Profs., DSc. UENF, Campos dos Goytacazes, RJ, Brazil. E-mail: claudia.marinho@uenf.br; pirapora@uenf.br; ricmsouza@uenf.br

³ Postdoctoral Researcher DSc. UENF, Campos dos Goytacazes, RJ, Brazil. E-mail: raudielle@outlook.com; joseolivio.agroecologia@yahoo.com.br

* Author for correspondence

Resumo

Um dos entraves ao melhoramento genético de plantas arbóreas é o tempo decorrido entre a seleção dos genótipos superiores e a multiplicação dessas variedades. Este estudo se concentra em híbridos de *Psidium*, desenvolvidos para obter porta-enxertos ou novos copas resistentes ao nematoide *Meloidogyne enterolobii*. O uso de meios-irmãos ou sementes de híbridos desses materiais genéticos não permite manter o perfil genético de indivíduos resistentes, tornando os métodos de seleção destrutivos imprevisíveis. A propagação por miniestaquia de material juvenil de *Psidium* resulta em alta porcentagem de enraizamento, facilitando a clonagem de famílias segregantes e reduzindo o tempo necessário para produzir réplicas. Neste estudo, famílias segregantes para resistência a *M. enterolobii* foram clonadas usando a miniestaquia, com réplicas mantidas em minijardins clonais enquanto as matrizes originais foram inoculadas e avaliadas para reprodução de nematoides no sistema radicular. Os resultados indicam segregação de resistência tanto entre quanto dentro das famílias. A clonagem precoce por miniestaquia demonstrou 100% de eficiência, permitindo que a identificação de 30 indivíduos resistentes ocorresse de forma simultânea ao primeiro ciclo de multiplicação desses indivíduos, reduzindo o tempo e a incerteza do resgate de materiais superiores. A metodologia, detalhada neste estudo, mostra-se uma estratégia eficaz, permitindo avanços para programas de melhoramento da goiabeira. Além disso, constata-se a presença de indivíduos resistentes a *M. enterolobii* em híbridos *P. guajava* x *P. cattleianum*; *P. cattleianum* x *P. guineense* e *P. guineense* x *P. cattleianum*.

Palavras-chave: Melhoramento da goiabeira. Miniestaquia. *Psidium cattleianum*. *Psidium guajava*. *Psidium guineense*.

Introduction

Guava is a socially and economically important crop in countries such as India, Pakistan, China and Brazil, the world's leading producers of the fruit (Altendorf, 2018). In Brazil, the Northeast and Southeast are known guava-producing regions, particularly the states of Pernambuco and São Paulo (Instituto Brasileiro de Estatística e Geografia [IBGE], 2022). Currently, the main phytosanitary challenge is controlling guava decline (Cardoso et al., 2017), a disease caused by the nematode *Meloidogyne enterolobii*, which colonizes the roots of guava trees, making them more vulnerable to attack by the fungus *Neocosmospora falciformes* (Gomes et al., 2011) and resulting in financial loss in different regions of Brazil

(Gomes et al., 2011; Castro et al., 2017; Souza et al., 2018).

Selecting resistant genotypes is a sustainable strategy for cultivation at nematode-contaminated sites or those at risk of contamination. *Psidium cattleianum* and *Psidium guineense* are considered sources of nematode resistance, leading to different crosses in pursuit of greater variability and easier selection of individuals for use as scions or rootstock (Carneiro et al., 2007; Biazatti et al., 2016; Castro et al., 2017). In *Psidium* species, natural propagation is generally seminiferous and by open pollination, which induces segregation of traits of interest. Studies show intraspecific variation in resistance to *M. enterolobii*, with susceptible or resistant genotypes found in

P. guineense and *P. cattleianum* (Costa et al., 2012; Miranda et al., 2012).

Using half-siblings or hybrid seeds would not preserve the same genetic profile of *M. enterolobii*-resistant individuals, making selection via destructive methods unfeasible. Gomes et al. (2017) multiplied genotypes prior to assessing resistance to *M. enterolobii*, preserving half of the root system for inoculation. However, this technique, generates greater uncertainty regarding the recovery of resistant materials and, at most, produces a single clone from each individual, making the multiplication of superior individuals more time-consuming, with a greater risk of losing material.

Propagation by minicutting juvenile *Psidium* material results in a high rooting percentage (Marinho et al., 2009; Arantes et al., 2021), and can be used to clone segregating families even before inoculation with the nematode.

The aim of this study was to evaluate the segregation of *M. enterolobii* resistance between and within families resulting from crosses between *P. guineense* x *P. cattleianum*, *P. guajava* x *P. cattleianum*, and *P. cattleianum* x *P. guineense*, through prior cloning of individuals by minicutting, as a strategy to shorten the time needed to obtain superior individuals with genetic reliability for the trait under study.

Material and Methods

Collection and treatment of genetic material

The genotypes evaluated in this study were derived from targeted crosses, as described and encoded by Gomes et al.

(2017). Initially, hybrids UENF 121 (*P. guajava* x *P. cattleianum*) and UENF 29 (*P. cattleianum* x *P. guineense*) were retrieved from the field through herbaceous cuttings, which were multiplied and maintained in two clonal minigardens. These progenitors then bore fruit and the seedlings obtained were used to establish two families (FA1 and FA2) in two new multiclonal minigardens. A third family (FA3) consisted of grafted 'Paluma' guava seedlings. The rootstocks of these seedlings were produced from a pool of parent plants of four hybrids (UENF 251, UENF 237, UENF 242, and UENF 253) that originated from *P. guineense* x *P. cattleianum* crosses and were kept in a multiclonal minigarden.

Obtaining Families FA1 and FA2

The seeds were treated and stored in a refrigerator at 5°C for planting, according to the protocol described by Robaina et al. (2012). The fruits of hybrid UENF 121 had a large number of small seeds (more than 90 per fruit), while those of UENF 29 contained a few large seeds (30 seeds per fruit). From 20 to 25 days after sowing (DAS), 145 seedlings were obtained for FA1 and 24 for FA2, and transplanted into 3 dm³ plastic bags at around four months old. The seedlings were pruned to stimulate new shoots for the production of minicuttings, in line with the procedure described by Altoé et al. (2011).

Production of minicuttings and acclimatization

The minicuttings were placed in 280 cm³ tubettes containing Basaplant® commercial substrate and then in a

nebulization chamber (15 s of nebulization at 10-minute intervals, at a flow rate of 7 L h⁻¹ and pressure of 4.0 kgf cm⁻²), where they remained for 60 days. After rooting, characterized by leaf maintenance, visible green coloring, and sprouting of minicuttings, the seedlings were acclimatized. The surviving seedlings were then transplanted into 3 L plastic bags, properly identified in the minigarden, and cultivated as clones of the individuals that were subsequently evaluated for nematode resistance.

Grafting of 'paluma' guava to obtain FA3

The seedlings used as rootstocks were obtained from a previously established multiclonal minigarden. Each hybrid was multiplied in successive collections of herbaceous cuttings. The minigarden consisted of a pool of four *Psidium* spp. hybrids (UENF 251, UENF 237, UENF 242, and UENF 253), originating from *P. guineense* and *P. cattleianum* crosses. Minicuttings were prepared from these materials and placed in 280 cm³ tubettes, according to the above protocol for rooting minicuttings from the other two families. The minicuttings were placed in the nebulization chamber for approximately 60 days and then acclimatized.

*Inoculation with *Meloidogyne enterolobii**

Inoculation with the nematode *M. enterolobii* was performed when FA1 and FA2 plants were eight months old, coinciding with eight months after grafting for FA3 plants. A pure *M. enterolobii* isolate was used as the

inoculum source. To prepare the inoculum, infected roots were submitted to modified extraction in accordance with the method described by Coolen and D'Herde (1972). The resulting suspension was adjusted to a concentration of 2,000 *M. enterolobii* eggs + second-stage juveniles (J2) in 10 mL of water, counted under a stereoscopic microscope using a Peter's slide. Seedlings were inoculated when they displayed six to eight pairs of leaves, each receiving 10 mL of the suspension, distributed in four holes around the root collar.

Experimental design

The families were compared using a completely randomized design (CRD), with three treatments corresponding to the *Psidium* families (FA1, FA2, and FA3), 12 replications, and two or four individuals per plot.

Extraction and counting of eggs and juveniles

Assessments were carried out at 135 days after inoculation, as proposed by Burla et al. (2010). For extraction of eggs and J2, plants were processed as previously described. The only modification was that the roots were shaken in an aqueous solution of 2 to 6% sodium hypochlorite instead of pure water. The suspension of eggs and J2 obtained from each plant was homogenized in three 1 mL aliquots and counted using a Peter's slide, expressed as the final nematode population (Pf).

Determining the reproduction factor (RF)

The reproduction factor (RF) was determined according to Oostenbrink (1966), by dividing the final population (number of eggs + J2 obtained from the root system of each plant) by the initial population (number of eggs + J2 inoculated). Final plant nematode resistance was classified based on the reproduction factor (RF = Final population / 2000), RF = 0 = immune, RF <1 = resistant, and RF >1 = susceptible (Oostenbrink, 1966).

Statistical analysis

The data were transformed due to non-normal distribution. Count data were transformed using $\sqrt{(x + 0.5)}$ for statistical analysis. The data were submitted to analysis of variance, and treatment means were compared by Tukey's test at 5% probability.

Results and Discussion

According to Oostenbrink (1966), of the 48 plants in FA1 (produced from the seeds of an individual resulting from the *P. guajava* x *P. cattleianum* cross), 14.6% were classified as resistant and 85.4% susceptible (Figure 1). These results corroborate those of Costa et al. (2012), who also observed significant susceptibility in *Psidium* crosses,

highlighting the complexity of genetic inheritance in resistance traits.

Of the 24 genotypes evaluated in FA2 (derived from *P. cattleianum* x *P. guineense*), 91.3% were classified as resistant and 8.7% susceptible (Figure 1), with the susceptible genotypes exhibiting a low reproduction factor (RF). This resistance is comparable to the findings of Carneiro et al. (2007), who observed significant prevalence of resistance in *Psidium* hybrids involving *P. cattleianum*. The strong genetic resistance in FA2 highlights *P. cattleianum* as a valuable donor of resistance traits, corroborating the results of Ribeiro et al. (2019).

Of the 48 genotypes assessed in FA3 (*P. guineense* x *P. cattleianum*), only one individual was deemed resistant, and 47 (97.9%) susceptible (Figure 1), although some obtained an RF only slightly higher than one. The highest RF (33.15) was recorded in this family, even greater than that of the 'Paluma' guava. This significant variability in FA3 suggests complex segregation of resistance traits, even when the hybrid involves contrasting individuals for resistance, as is the case for the progenitors of the families studied here. Resistance in *P. cattleianum* accessions was initially investigated by Biazatti et al. (2016), and these genotypes later became the progenitors of the hybrids evaluated in the present study.

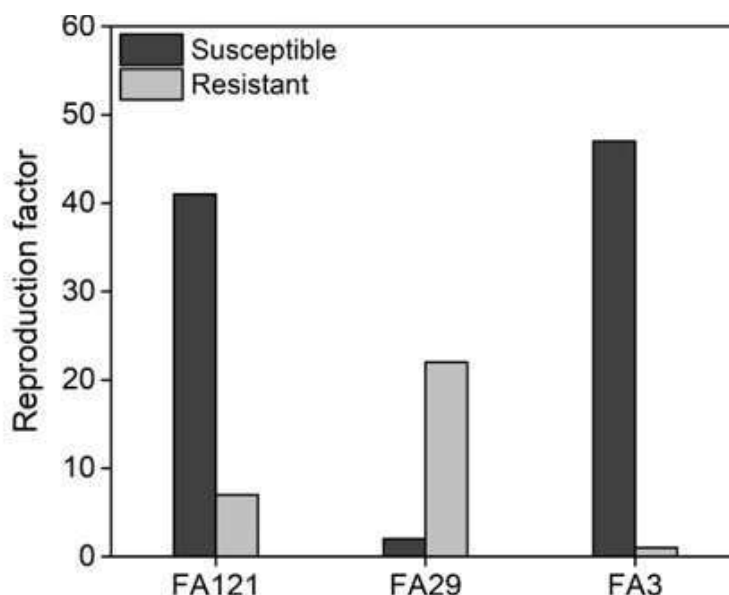


Figure 1. Number of individuals resistant or susceptible to *M. enterolobii* in families (FA) 1, 2 and 3, in accordance with Oostenbrink (1966).

The difference in RF within the three families demonstrates that the presence of a resistant individual as a progenitor (*P. cattleianum*), as is the case for all the crosses evaluated here, did not always result in high resistance frequency in the progenies analyzed. This reinforces the need to clone resistant individuals for use as rootstock to prevent resistance segregation from interfering with genetic gains for *M. enterolobii* resistance.

The 'Paluma' cultivar is characterized as susceptible to *M. enterolobii*, which was confirmed in the present study and validates previous findings by Gomes et al. (2017), emphasizing the cultivar's vulnerability and the reliability of the inoculation methods used.

Data on the number of eggs per gram of root (NE/G) and reproduction

factor (RF) are shown in Table 1. The lowest NE/G and RF values and highest RF were recorded in the FA2 family, derived from the fruits of an individual obtained from the *P. cattleianum* x *P. guineense* cross, the family with the highest indicators of *M. enterolobii* resistance. Oliveira et al. (2019) also reported RF variations in *Psidium* genotypes, demonstrating the importance of genetic diversity in guava breeding programs.

M. enterolobii parasitism in the root system of *Psidium* plants causes galls, which contain a mass of the pathogen's eggs (Fachinello et al., 2000). In the present study, different gall levels were observed in the root systems of *Psidium* families. While 'Paluma' guava and susceptible plants from FA1 and FA3 showed significant gall formation, some individuals exhibited few or no galls; however, lack of galls is not the sole factor in confirming resistance.

Table 1

Number of eggs/gram of root and reproduction factor in genotypes of three *Psidium* families 135 days after inoculation with *M. enterolobii*; FA1 and FA 2 at 260 days after emergence and FA3 at 500 days after grafting onto rootstock

Family	Number of eggs/gram of root	Reproduction Factor
FA1	127.4 a	8.21 a
FA2	14.1 b	0.47 b
FA3	186.1 a	8.02 a
CV (%)	34.26	14.82

Mean values followed by the same lowercase letter in the column for the same trait do not differ according to Tukey's test at 5% probability. CV = coefficient of variation.

All the 'Paluma' guava individuals displayed a large number of galls, with high RF values of 10.49 to 27.95, confirming their

susceptibility (Figure 2) previously reported by Biazatti et al. (2016) and Gomes et al. (2017).



Figure 2. Root system of individuals from *Psidium* families evaluated 135 days after inoculation with *M. enterolobii*: A = FA2; B = Guava 'Paluma'; C = FA3 with few galls; D = FA3 with a large number of galls; E = FA1, with few galls; F = FA1, with a large number of galls.

Based on Oostenbrink's (1966) criteria, 30 individuals were classified as resistant to *Meloidogyne enterolobii* colonization. Ribeiro et al. (2019) reported similar results in accessions from *P. guajava* x *P. cattleianum* crosses, emphasizing the feasibility of incorporating resistance through interspecific hybridization.

Ribeiro et al. (2019) demonstrated the feasibility of interspecific hybridization in breeding aimed at incorporating *M. enterolobii* resistance into new scion or rootstock options for guava. This was confirmed in the present study, where resistance was also observed in families originating from the self-fertilization of flowers from these hybrids. The present study demonstrates the potential of self-fertilization in selecting resistant individuals, corroborating the findings of V. M. Freitas et al. (2014) and Gomes et al. (2017), who highlighted the lack of resistance in guava cultivars (*P. guajava*).

The present study indicates resistance segregation when hybrids are propagated by seeds, thus reinforcing the need for vegetative propagation of individuals before selecting for nematode resistance. This approach ensures the preservation and multiplication of resistant genotypes, as observed by Altoé et al. (2011) and Marinho et al. (2009), who demonstrated the effectiveness of minicutting techniques.

The minicutting technique used for genotype propagation in this experiment made it possible to maintain clones of

all individuals from the three families in clonal minigardens. As a result, the plants identified as resistant were preserved with no risk of losing resistance alleles, and since multiplication and selection were carried out simultaneously, more of these individuals were obtained in a shorter time period. J. A. A. Freitas et al. (2013) reported similar success rates, supporting the use of minicutting for rapid and efficient propagation of genetic material during its juvenile phase.

The proposed methodology is represented in Figure 3, in a timeline showing the necessary steps to select and multiply resistant material, saving time and preserving genetic gains. In the clonal minigardens used in the present study, each genotype had more than one clone 375 days after sowing of the hybrids, thereby accelerating the subsequent steps of multiplying superior individuals and discarding their susceptible counterparts. Minicuttings of juvenile *Psidium* material have been successfully used for seedling production, always producing a high rooting percentage, with 90 to 100% efficiency (Marinho et al., 2009; Altoé et al., 2011), demonstrating its potential as an auxiliary tool in *Psidium* breeding. This multiplication effectiveness was also reported by Arantes et al. (2021), who obtained high rooting percentages using similar propagation techniques in the *Psidium* hybrid 'BRS Guaraçá'. In the present study, the technique resulted in 100% efficiency in multiplying genotypes before selection for nematode resistance assessment.

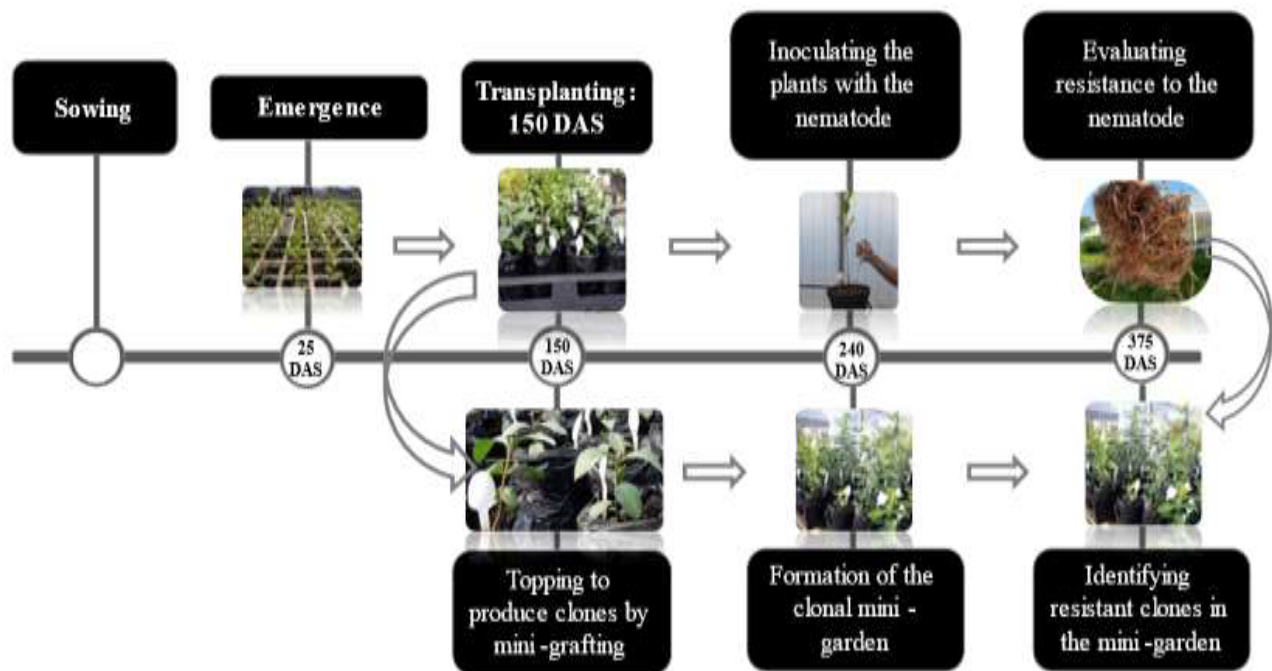


Figure 3. Flowchart of the proposed methodology for accelerated production of *M. enterolobii*-resistant individuals, from sowing to the identification of resistant material in the clonal minigarden at 375 DAS (DAS - days after sowing).

The high efficiency of this type of propagation requires an environment with strict humidity controls inside the spray chamber, to enable the acclimatization and establishment of the rooted cuttings. Environmental data from inside the greenhouse are shown in Figure 4. At night, relative humidity and minimum average temperatures were close to 90% and 20°C, respectively, whereas daytime humidity did

not approach saturation, mainly due to the higher daytime temperatures. Nevertheless, the high rooting percentage recorded here demonstrates that these conditions were adequate for the minicutting propagation of juvenile material. Similar results were obtained by J. A. A. Freitas et al. (2013), who also observed successful rooting of three guava varieties under controlled humidity and temperature conditions.

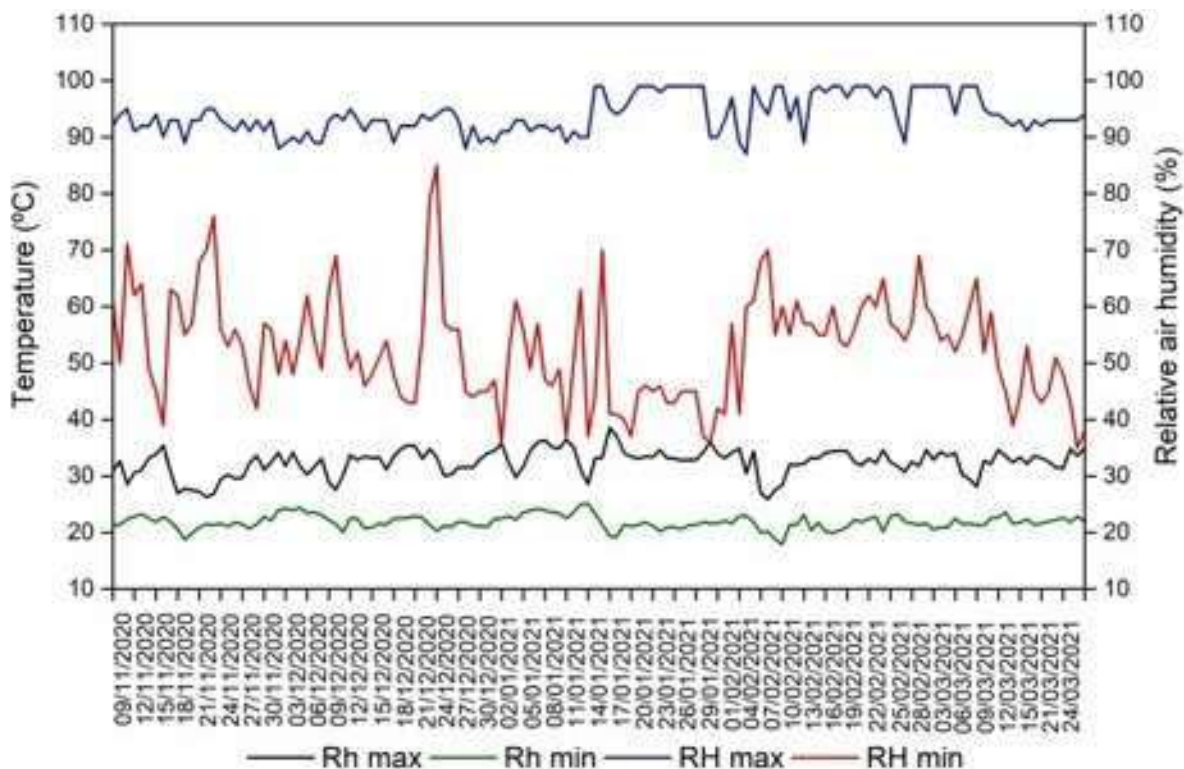


Figure 4. Average maximum and minimum temperatures (T max and T min) and relative humidity (RH) inside the greenhouse between February 2020 and March 2021.

When considering genotypes for use as rootstocks, in addition to *M. enterolobii* resistance, graft compatibility of the selected genotype with guava should also be a requirement. As such, genotypes from families with phenotypes closer to guava, such as those from FA1, derived from self-fertilization of *P. guajava* x *P. cattleianum* hybrids, are expected to have greater graft affinity with guava, which should be investigated in future studies, as suggested by previous research (Marinho et al., 2009; Altoé et al., 2011).

Conclusions

Segregation of resistance to *Meloidogyne enterolobii* occurs both in individuals obtained by self-fertilization and in half-siblings of those classified as resistant. Cloning by minicutting in juvenile material, performed before inoculation with the nematode, results in 100% recovery of segregating families and, when carried out simultaneously with resistance assessment, reliably preserves the selected individuals and accelerates the first multiplication cycle of superior genotypes. *M. enterolobii*-resistant individuals are found in segregating families derived from *P. guajava* x *P. cattleianum*, *P. cattleianum* x *P. guineense*, and *P. guineense* x *P. cattleianum* crosses.

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