https://doi.org/10.1590/1678-4499.20180058

PLANT PROTECTION - Article

Characterization of tolerance to citrus leafminer of *Citrus* and related genera

Mônica Santos[1], Rafael Major Pitta[2], Leandro do Prado Ribeiro[3], Nivia Silva Dias-Pini[4]*, José Djair Vendramim[5]

1.Escola Superior de Agricultura "Luiz de Queiroz" - Departamento de Entomologia e Acarologia - Laboratório de Resistência de Plantas e Plantas Inseticidas -Piracicaba (SP), Brazil.
2.Embrapa Agrossilvipastoril - Laboratório de Entomologia - Sinop (MT), Brazil.
3.Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina - Centro de Pesquisa para Agricultura Familiar - Chapecó (SC), Brazil.
4.Embrapa Agroindústria Tropical - Laboratório de Entomologia - Fortaleza (CE), Brazil.
5.Escola Superior de Agricultura "Luiz de Queiroz" - Departamento de Entomologia e Acarologia - Piracicaba (SP), Brazil.

ABSTRACT: The aim of this study was to identify resistance of tolerance type in *Citrus* and *Poncirus* genotypes towards the citrus leafminer (CLM), *Phyllocnistis citrella*. The quantitative variables leaf and shoot lengths, leaf width, number of larvae and new shoots, and fresh and dry shoots weights, and qualitative variables associated with foliar damage were evaluated in six *Citrus*-related genotypes infested with CLM. In preliminary trials with lime Rough lemon (*C. jambhiri*), the variables that best discriminated the infestation effect of CLM were established as the numbers of larvae and new shoots per plant together with the percentages of partially rolled leaves (PRL), rolled leaves (RL) and total damage (TD = RL + PRL). In subsequent tests with all six genotypes, the variables new shoots per plant, percentage of attacked but not rolled leaves (ANRL), RL and TD were found to be significant. Trifoliata Limeira (*P. trifoliata*) and hybrid C × R_4 (*C. sunki* × *P. trifoliata*) presented the lowest percentages of RL and TD and the highest values of ANRL. A cluster analysis was performed considering all the variables analyzed and the most tolerant genotypes for CLM, namely hybrids C × R_4, C × R_{315} (*C. sunki* × *P. trifoliata*), M × P_{222} [*C. sinensis* × Tangor Murcott (*C. reticulata* × *C. sinensis*)] and Trifoliata Limeira (*P. trifoliata*) were grouped apart from the less tolerant genotypes Sunki mandarin (*C. sunki*) and lime Rough lemon. In conclusion, genotypes Trifoliata Limeira and its hybrid C × R_4 are the most tolerant to CLM.

Key words: *Phyllocnistis citrella*, resistance categories, host selection, sustainable pest management, *Poncirus* genotypes.

*Corresponding author: nivia.dias@embrapa.br

Received: Feb. 16, 2018 – **Accepted:** Nov. 12, 2018

INTRODUCTION

The citrus leafminer (CLM), *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), is a pest of considerable importance to citriculture worldwide (Mustafa et al. 2014). The adult females oviposit preferentially on the abaxial surfaces of young leaves. Immediately after hatching, the larvae penetrate the foliar tissue quickly breaking, remaining within the leaf mine throughout their developmental period (Willink et al. 1996). The biological cycle of MLC depends on the temperature, requiring 32.7 days from egg to adult at 18 °C. Another factor that affects the cycle is citrus variety (Chagas and Parra 2000). Direct damage caused by CLM results in the reduction in leaf area, premature leaf fall and reduction in shoot development, thereby compromising photosynthetic activity and, consequently, crop productivity (Heppner 1993). In addition, the extensive injuries caused by CLM facilitate the ingress of microorganisms, particularly of the bacterium *Xanthomonas citri* subsp. *citri*, the causal agent of citrus canker (Hall et al. 2010).

The leaf-mining habit of *P. citrella* renders it hard to control because of the difficulty in reaching larvae inside leaf tissue. In Brazil, the primary method of control employs synthetic insecticides that are generally applied by spray virtually all year round in orchards in the main producing regions (Paiva 2011). However, exposure to insecticides can lead to ecological imbalance, while their frequent use exerts high selection pressure, such that populations of *P. citrella* resistant to some classes of chemicals have already been found (Morais et al. 2016). The ensuing reduction in efficiency of the control agents often necessitates an increase in dose or number of applications, thereby enhancing the negative effects.

It is necessary to integrate control strategies within an integrated management program (IPM), including importation of parasitoid *Ageniaspis citricola* Logvinovskaya (Hymenoptera: Encyrtidae) (Hoy et al. 2007), selection of native natural enemies (Goane et al. 2015) and the sexual confusion technique with synthetic pheromone (Stelinski et al. 2008). Following the introduction of *A. citricola* in Brazil, this parasitoid became widespread and settled in 100% of citrus areas, significantly reducing larval infestations of the pest. *A. citricola*'s parasitism rates averaged 40%, reaching rates higher than 90% in some localities (Chagas and Parra 2000). In addition, the use of resistant cultivars represents an attractive strategy for the management of pest species, especially for perennial crops.

Studies have been conducted to detect sources of resistance in *Citrus* genotypes and other related genera (Goane et al. 2008; Santos et al. 2011), and factors of resistance to *P. citrella* involving antibiosis and antixenosis mechanisms have been identified. However, no reports are currently available concerning the evaluation of resistance of tolerance type to *P. citrella*. In light of the above we tested, under laboratory conditions, the hypotheses that there is tolerance among *Citrus* genotypes and related genera that is associated with resistance to *P. citrella*.

MATERIAL AND METHODS

Adult CLMs used in the assays came from a population reared and maintained under controlled laboratory conditions (26 ± 1 °C, relative humidity 70 ± 10% and 14 h photophase) in aluminum cages (50 × 50 × 50 cm) arranged on metal shelves. The upper, back and side walls of the cages were covered with anti-aphid white screen, while the front walls consisted of two 50 × 25 cm acrylic panels that could be removed to allow the handling and removal of plants. One of the front panels of each cage incorporated a 20 cm diameter opening equipped with a voile fabric sleeve through which insects could be released or retrieved (Figs. 1a and 1b). Seedlings of Rangpur lime (*Citrus limonia* Osbeck) were provided as substrate for feeding and oviposition.

The genotypes evaluated were Sunki mandarin (*C. sunki* hort. ex Tanaka), hybrids C × R_4 [*C. sunki* × *Poncirus trifoliata* (L.) Raf], C × R_{315} (*C. sunki* × *P. trifoliata*) and M × P_{222} [*C. sinensis* Osbeck × tangor Murcott (*C. reticulata* Blanco × *C. sinensis*)], along with Trifoliata Limeira (*P. trifoliata*), which was previously selected as resistant to CLM (Santos et al. 2011) and Rough lemon (*C. jambhiri* Lush.), which presents a typical susceptibility pattern. Prior to the tests, plants of these genotypes were multiplied by grafting and cultivated in plastic bags (3.8 L) with commercial substrate consisting of composted pine bark and vermiculite (Plantmax®, Eucatex Mineral, Paulínia, SP, Brazil). Seedlings were kept in a greenhouse where they were pruned at 1/3 from the apex and fertirrigated twice a week with a nutrient solution prepared according to the recommendations of the Centro de Citricultura →

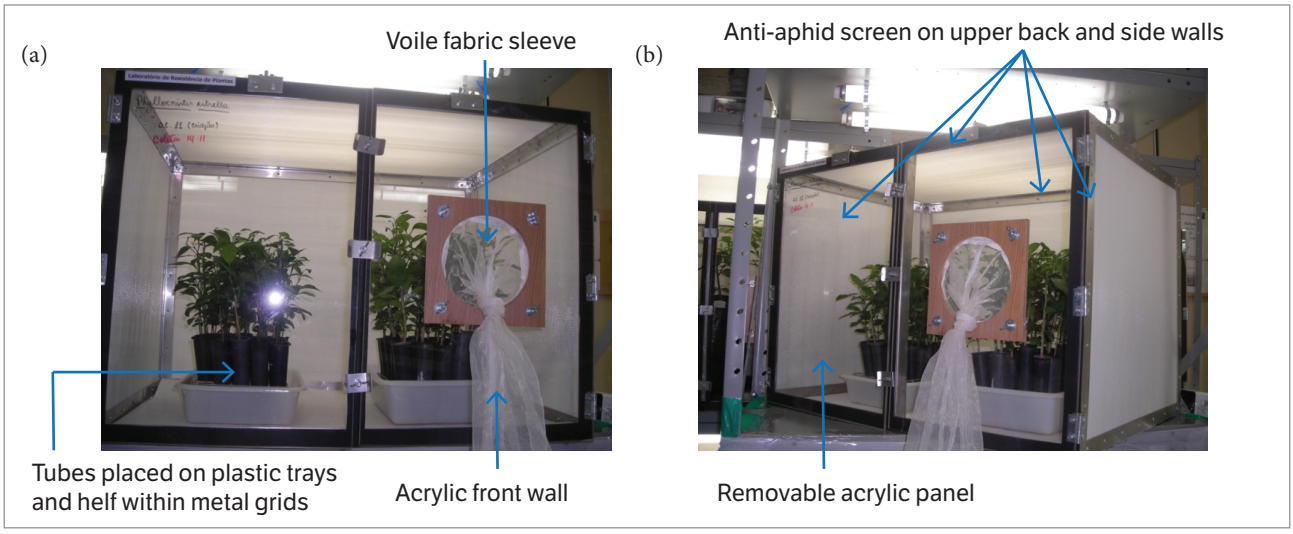

Figure 1. Cages used in rearing *Phyllocnistis citrella* under laboratory conditions showing (a) front view, and (b) side view.

Sylvio Moreira (Cordeirópolis, SP, Brazil). The plants used in the tests were approximately 50 cm height. All bioassays were conducted under controlled conditions (26 ± 1 °C, relative humidity 70 ± 10%, and 14 h photophase) and performed using a completely randomized experimental design.

Determination of infestation density and discriminant variables

Preliminary tests were carried out using plants of Rough lemon in order to determine the infestation density required to cause significant damage and to select the best parameters by which to assess tolerance of genotypes to CLM. Plants showing new growth were selected and four shoots with standardized dimensions (8 to 10 cm long) were marked at their bases with permanent ink (Pilot Pen do Brasil, São Paulo, SP, Brazil). Plants were placed in individual aluminum cages (described above) and adult CLMs of up to 48 h of age were separated by sex and released at different densities (0, 3, 6, 9 and 12 couples per plant) into the cages. The experimental unit comprised one plant (infested with eggs) maintained inside the cage for 13 days, and four repetitions were performed at each level of treatment.

During the assay period, plants were assessed with regard to: (a) quantitative variables represented by shoot length, leaf length and width, number of larvae per leaf, number of new shoots per plant, number of leaves per shoot, and fresh and dry weights of shoots; and (b) qualitative variables related to damage to the leaves. Changes in shoot growth associated with different infestation levels were determined by measuring the lengths of the selected shoots on the 1st (initial) and 13th (final) days of the assay. Lengths and widths of leaves were evaluated on the 1st and 8th days, since leaves started to roll from the 9th day onward and this could hamper assessment. The numbers of larvae on the adaxial and abaxial surfaces of leaves were established on the 5th day after infestation, while the number of leaves per shoot and number of new shoots per plant were assessed on the 13th day. Weights of individual shoots were determined using an analytical balance with 0.1 mg accuracy. Fresh weight was assessed immediately after shoot excision on the 13th day, while dry weight was established after the cut shoot had been maintained in an oven at 40 °C for 48 h. Leaf damage was assessed by visual inspection according to the following classification: attacked but not rolled leaves (ANRL), partially rolled leaves (PRL), and rolled leaves (RL) (Figs. 2a, 2b and 2c).

Evaluation of tolerance of the genotypes

After the number of insects and the appropriate discriminant variables to be used in the bioassays had been determined, tests were conducted to evaluate tolerance type resistance in the six studied genotypes. In these assays, plants of each genotype were placed in individual cages and six CLM couples were released for oviposition. Insects were removed from the cages after 3 days, and on the 5th day the larvae on each plant were counted and any excess removed with the aid of a stylet to leave 20 larvae per plant. The plants were replaced in the cages and on the 13th

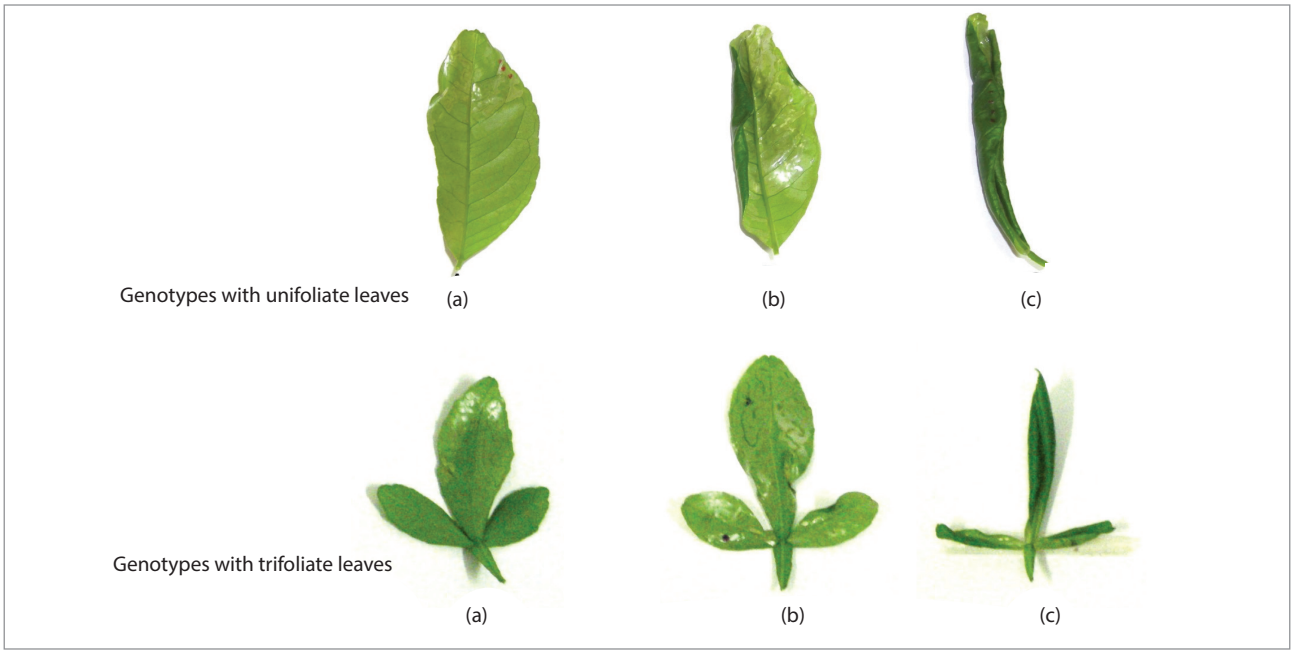

Figure 2. Effects of *Phyllocnistis citrella* attack on *Citrus* and *Poncirus* genotypes with unifoliate or trifoliate leaves showing (a) attacked but not rolled leaf (ANRL), (b) partially rolled leaf (PRL), and (c) rolled leaf (RL).

day, the numbers of new shoots were determined and the leaf damage assessed according to the criteria adopted in the preliminary test with Rough lemon. For the trifoliate genotypes, i.e. Trifoliata Limeira and hybrids C × R_4 and C × R_{315}, evaluations were made considering the entire leaf, while for the three unifoliate genotypes evaluations were made for each leaflet. Five repetitions were performed for each treatment and each repetition consisted of one plant. In order to allow for differences in growth rates between genotypes, non-infested (control) plants of each genotype were subjected to bioassay for comparison purposes.

Data analysis

A pre-adjustment model was initially performed assuming normal distribution of data and the normality of residuals and homogeneity of variances were tested using Shapiro-Wilk (Shapiro and Wilk 1965) and Bartlett (Bartlett 1937) tests. When the data did not show normality and/or homoscedasticity, transformations were carried out based on the Box-Cox optimal power method (Box and Cox 1964). Treated data were subjected to analysis of variance (ANOVA) and the mean values of qualitative variables determined at different treatment levels were compared using the Tukey test. The relationships between quantitative variables and treatment levels were examined using a linear regression approach and the goodness of fit was verified in each case. Data obtained from the assessment of the number of new shoots for different genotypes were compared using the Student *t* test for independent samples. All analyses were performed using the R statistical software version 3.2.3 with significance levels set at 0.5.

Multivariate analyses were performed to verify the clustering of genotypes based on all (qualitative and quantitative) of the variables studied. For this purpose, the Euclidean distance was employed as a dissimilarity measurement with the unweighted pair group method with arithmetic mean (UPGMA) as the grouping strategy. These analyses were carried out with the aid of STATISTICA software version 6.0.

RESULTS

Preliminary tests with Rough lemon (Table 1) revealed significant effects of CLM infestation density on the variables number of larvae per plant ($y = 3.384x + 0.176$, $R^2 = 0.385$, $p = 0.006$) and number of new shoots per plant ($y = -0.2431x + 1.167$, $R^2 = 0.314$, $p = 0.006$), with both variables showing linear increases according to the increasing →

number of couples per plant. On the other hand, the variables leaf length and width, shoot growth and fresh and dry weights of shoots were not affected by infestation, even at the highest levels. Regarding the qualitative parameters, increases in infestation density produced significant effects on the percentages of PRL ($y = 3.55x + 0.492$, $R^2 = 0.26$, $p = 0.02$) and RL ($y = 1.40x + 1.117$, $R^2 = 0.41$, $p = 0.002$) and, consequently, of TD ($y = 4.95x + 1.61$, $R^2 = 0.51$, $p < 0.001$) (Table 2).

Consideration of the quantitative and qualitative variables analyzed indicated that six CLM couples per plant were sufficient to cause significant damage to Rough lemon and provided a uniform level of infestation of 20 larvae per plant. Furthermore, the variables that best discriminated the effects of CLM infestation were the number of new shoots per plant and the percentages of ANRL, PRL, RL and TD.

The mean numbers of new shoots produced by CLM infested and non-infested plants of the six studied genotypes (Table 3) revealed that the presence of the pest induced a significant reduction in new shoot formation only in the hybrid $C \times R_{315}$. Evaluation of qualitative variables for CLM infested plants of the six studied genotypes (Table 4) showed that the percentage of ANRL in Trifoliata Limeira was significantly higher than in genotypes Sunki mandarin, Rough lemon and hybrid $M \times P_{222}$, but not significantly different from the values determined in hybrids $C \times R_4$ and $C \times R_{315}$. In contrast, the percentages of RL and TD showed an essentially inverse pattern in which the lowest values were recorded in Trifoliata Limeira and the highest values in Sunki mandarin and Rough lemon with intermediate values in the three hybrid genotypes. No significant differences were detected between the genotypes in relation to the percentages of PRL.

Hierarchical cluster analysis based on all variables analyzed (Fig. 3) indicated that the genotypes formed two groups. The first group comprised the less tolerant genotypes Sunki mandarin and Rough lemon, while the second group contained the remaining four genotypes, which showed tolerance or intermediate behavior. The more tolerant →

Table 1. Mean values (± SE) of leaf length (LL), leaf width (LW), shoot growth (SG), number of larvae per plant, number of new shoots per plant, and fresh and dry weight of shoots of Rough lemon according to level of infestation by *Phyllocnistis citrella* adults.

Number of couples per plant	Quantitative variables						
	LL (cm) (F – I)[a]	LW (cm) (F – I)[a]	SG (cm) (F – I)[a]	Number of larvae per plant	Number of new shoots per plant	Fresh weight (g)	Dry weight (g)
0	4.4 ± 0.43	2.9 ± 0.78	10.9 ± 2.18	0.0 ± 0.00	3.3 ± 0.58	2.8 ± 0.41	0.5 ± 0.11
3	3.7 ± 0.28	2.6 ± 0.62	8.5 ± 1.13	12.8 ± 2.19	4.0 ± 0.41	2.2 ± 0.65	0.3 ± 0.09
6	3.2 ± 0.20	2.3 ± 0.34	9.9 ± 1.52	18.9 ± 4.59	6.5 ± 0.87	3.1 ± 0.53	0.6 ± 0.15
9	3.4 ± 0.35	2.4 ± 0.63	10.6 ± 1.12	27.7 ± 7.06	6.5 ± 0.96	1.9 ± 0.37	0.4 ± 0.10
12	3.6 ± 0.39	2.5 ± 0.72	9.4 ± 1.81	34.1 ± 6.37	6.5 ± 0.87	2.3 ± 0.17	0.5 ± 0.06
F	1.29	0.96	0.01	10.38	9.70	0.68	0.05
p	0.27	0.34	0.92	0.01	0.01	0.42	0.81

[a] F = Final; I = Initial.

Table 2. Mean values (± SE) of the percentage of attacked but not rolled leaves (ANRL), partially rolled leaves (PRL), rolled leaves (RL) and total damage (TD = RL + PRL) assessed in Rough lemon according to level of infestation by *Phyllocnistis citrella* adults.

Number of couples per plant	Qualitative variables			
	ANRL (%)	PRL (%)	RL (%)	TD (%)
0	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
3	30.5 ± 6.76	29.9 ± 3.15	15.2 ± 2.42	45.1 ± 2.39
6	14.5 ± 2.98	28.2 ± 2.20	32.5 ± 9.44	60.6 ± 8.54
9	18.3 ± 6.72	28.9 ± 10.13	36.2 ± 14.76	65.0 ± 10.37
12	21.5 ± 11.79	26.1 ± 2.50	46.2 ± 7.51	72.3 ± 8.90
F	1.65	6.47	12.93	18.55
p	0.22	0.02	0.002	< 0.001

Table 3. Mean values (± SE) of the number of new shoots per plant on *Citrus* and *Poncirus* genotypes without infestation and with infestation by six couples of *Phyllocnistis citrella* and standardized at 20 larvae per plant.

Genotypes	Number of new shoots per plant*			
	Without infestation	With infestation	Student t	p
Rough lemon	4.0 ± 0.32A	3.8 ± 1.11 A	0.17	0.87
Sunki mandarin	2.8 ± 0.37A	3.0 ± 0.95 A	0.20	0.85
Hybrid C × R4	1.6 ± 0.51A	2.6 ± 1.03 A	0.87	0.42
Hybrid C × R315	5.4 ± 1.03B	2.4 ± 0.81 A	2.29	0.05
Hybrid M × P222	2.8 ± 0.86A	2.0 ± 0.63 A	0.75	0.48
Trifoliata Limeira	1.4 ± 0.40A	1.2 ± 0.73 A	0.24	0.82

* In each row, mean values bearing similar upper case letters are not significantly different according to the Student t test for independent variables ($p > 0.05$).

Table 4. Mean values (± SE) of the percentage of attacked but not rolled leaves (ANRL), partially rolled leaves (PRL), rolled leaves (RL) and total damage (TD = RL + PRL) assessed in *Citrus* and *Poncirus* genotypes infested by six couples of *Phyllocnistis citrella* and standardized at 20 larvae per plant.

Genotypes	Qualitative variables*			
	ANRL (%)[a]	PRL (%)[a]	RL (%)[a]	TD (%)[a]
Trifoliata Limeira	44.0 ± 5.34A	31.0 ± 6.60	21.0 ± 6.96B	52.0 ± 4.06C
Hybrid C × R4	36.0 ± 6.78AB	25.0 ± 6.12	31.0 ± 9.92AB	56.0 ± 5.34BC
Hybrid C × R315	26.0 ± 8.72AB	32.0 ± 3.74	39.0 ± 6.04AB	71.0 ± 9.54ABC
Rough lemon	12.0 ± 3.74B	26.0 ± 6.20	58.0 ± 3.74A	84.0 ± 4.00AB
Hybrid M × P222	10.0 ± 3.16B	16.0 ± 3.67	41.0 ± 14.35AB	57.0 ± 11.90BC
Sunki mandarin	10.0 ± 4.18B	24.0 ± 5.79	67.0 ± 3.74A	91.0 ± 4.00A
F	4.82	1.20	3.48	4.61
p	0.003	0.338	0.016	0.004

*In each column, mean values bearing similar upper case letters are not significantly different according to the Tukey test ($p > 0.05$). [a]Original data presented in the Table. For analysis, data were subjected to $\sqrt{X + 0.5}$ transformation.

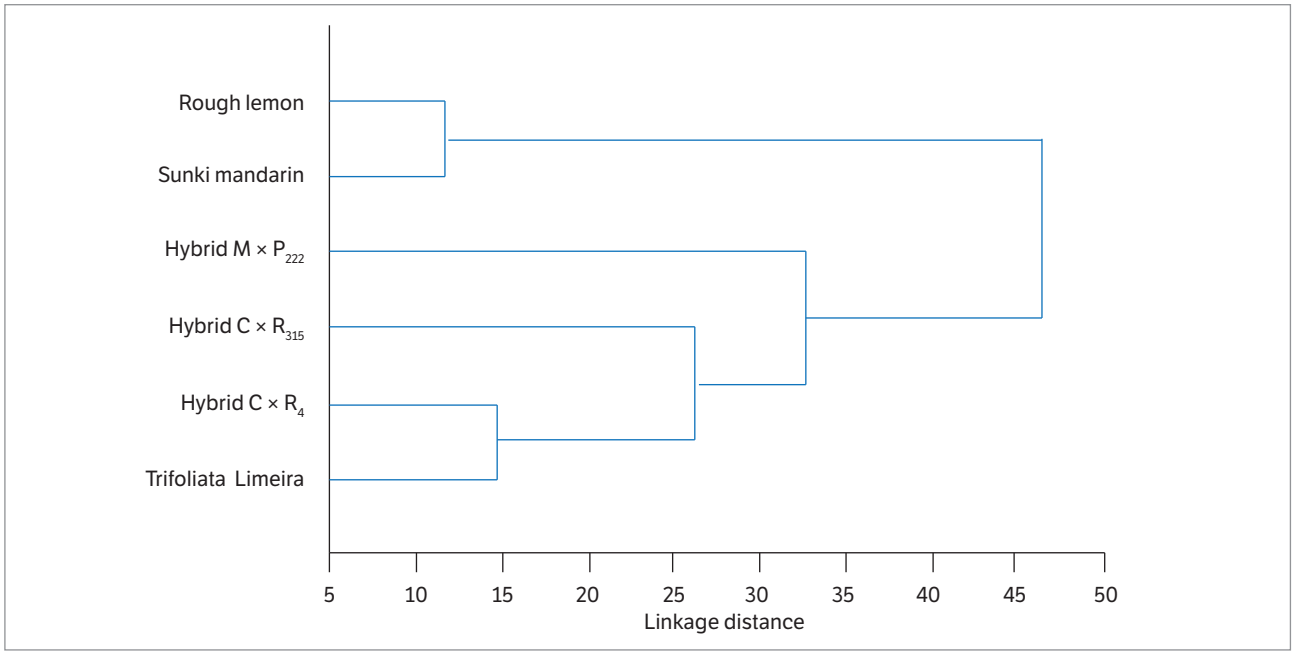

Figure 3. Dendrogram obtained through cluster analysis based on quantitative and qualitative variables assessed in *Citrus* and *Poncirus* genotypes infested by *Phyllocnistis citrella*. Euclidean distance was employed as a dissimilarity measurement with the unweighted pair group method with arithmetic mean (UPGMA) as the grouping strategy.

Trifoliata Limeira rootstock and its hybrid C × R_4 presented the greatest Euclidean distances between the genotypes tested, considering all variables studied.

DISCUSSION

Plant tolerance can be described as the extent to which a plant can support an insect infestation without loss of vigor and reduction of crop yield. Plant tolerance is usually taken to mean that when two cultivars are equally infested the less tolerant one has a smaller yield (Dent 2000).

Citrus species, hybrids and varieties can produce new flushes continuously after foliar damage, stress or pruning. The yield losses depend on the rootstock, physiological stage of trees, nutritional conditions, climate and water available. The parameters described in the present paper may be only a group of the components of the possible tolerance.

According to Sadasivam and Thayumanavan (2003), comparison of damage caused, the proportion of surviving plants and the amount of biomass produced in infested and non-infested specimens of different cultivars are important factors in determining resistance of the tolerance type.

In the present study, the increased numbers of CLM larvae per plant observed with increasing infestation density was not unexpected and provided evidence that, within the density range of 3 to 12 couples per plant, there was no competition between females for egg-laying substrate.

Assays conducted to evaluate the tolerance of six genotypes using pest densities and discriminatory variables established in previous tests revealed that the number of new shoots per plant increased with increasing infestation density for all genotypes except hybrid C × R_{315}. This finding likely indicates a strategy adopted by the majority of the studied genotypes to offset the damage suffered by insect attack. According to Tiffin (2000), one of the major physiological responses associated with herbivory in plants involves compensatory growth, and the emission of new shoots or tillers is known to confer resistance of the tolerance type to pest attack (Martins et al. 1977; Lara 1991). Hybrid C × R_{315} infested plants, which produced fewer new shoots than their non-infested counterparts, may have embraced mechanisms to decrease pest attack involving reduction in the emission of new shoots, thus hindering CLM proliferation, and shedding infested shoots (a hypersensitivity reaction). According to Strauss and Agrawal (1999), the mechanisms underlying tolerance may differ according to the feeding mode of herbivores as, for example, in cotton plants (*Gossypium hirsutum*) that showed increased branching in response to bud removal but decreased branching in response to aphid attack.

Santos et al. (2011) demonstrated that hybrids C × R_{315} and C × R_4 have CLM resistance factors of the antibiosis type. Given that these hybrids are derived from the crossing *C. sunki* × *P. trifoliata* and that the latter, according to the genetic analysis performed by Bernet et al. (2005), presents a resistance gene that causes antibiosis, it is inferred that this genetic basis of resistance may have been transmitted to the hybrids.

Leaf rolling is the main symptom of pest attack (Nascimento et al. 2000). In the present study, the symptom was well evident in Rough lemon and Sunki mandarin, indicating the susceptibility of these genotypes. Leaf rolling is the qualitative variable most widely used to determine leaf damage by CLM and the percentage of damaged area (Knapp et al. 1993; Jacas et al. 1997), although other methods, such as image analysis, have been applied to assess the accuracy and variability of qualitative estimates (Schaffer et al. 1997). In the present study, ANRL, RL and TD were the leaf damage variables that best discriminated the genotypes with regard to tolerance to CLM attack. In contrast, PRL did not appear to be useful for damage assessment, and consequently, for the determination of varietal tolerance.

Tiffin (2000) reports that the identification of tolerance mechanisms requires, in addition to studies of the physiological and morphological changes that occur after herbivory, investigations into the relationship between characteristics and the expression of tolerance.

Further studies are needed to assess the potential of these genotypes to herbivory tolerance of CLM for a better understanding of possible mechanisms involved in tolerance expression.

ACKNOWLEDGMENTS

We acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarships to the first and the last authors. We thank Dr. André Luiz Lourenção (Instituto Agronômico de Campinas)

for his support in the selection of genotypes tested and valuable suggestions for the study, and Dr. Mariângela Cristofani Yaly and employees Benedito Vanderley da Cunha and Osvaldo Betti (Instituto Agronômico de Campinas) for donation, maintenance and grafting of seedlings.

AUTHORS' CONTRIBUTION

Conceptualization, Santos M. and Vendramim J. D.; Methodology, Santos M.; Pitta R. M. and Ribeiro L. P.; Investigation, Santos M., Pitta R. M., Ribeiro L. P. and Dias-Pini N. S.; Writing – Original Draft, Santos M., Pitta R. M., Ribeiro L. P. and Dias-Pini N. S.; Writing – Review and Editing, Santos M. and Dias-Pini N. S.; Funding Acquisition, Santos M. and Vendramim J. D.; Resources, Vendramim J. D.; Supervision, Vendramim J. D.

ORCID IDs

M. Santos
https://orcid.org/0000-0001-9398-806X
R. M. Pitta
https://orcid.org/0000-0002-8503-7106
L. P. Ribeiro
https://orcid.org/0000-0002-7846-5620
N. S. Dias-Pini
https:/orcid.org/0000-0002-3664-812X
J. D. Vendramim
https://orcid.org/0000-0003-3122-4691

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