

# Neotropical Entomology

ISSN: 1519-566X

journal homepage: [www.scielo.br/ne](http://www.scielo.br/ne)

## PEST MANAGEMENT

### Resistance of Citrus Genotypes to *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae)

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#### Keywords

Citrus leafminer, host plant, biology

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Edited by Antônio R Panizzi – EMBRAPA

Received 11 January 2010 and accepted 15 March 2011

#### Abstract

The development and reproduction of the citrus leafminer (CLM), *Phyllocnistis citrella* Stainton, were evaluated in six citrus genotypes in order to identify genotypes with resistance traits that could be applied in a program for the development of citrus varieties resistant to the citrus leafminer. Tests were conducted under controlled laboratory conditions ( $25 \pm 1^\circ\text{C}$ ,  $70 \pm 10\%$  RH, and 14h photophase). Seedlings of each genotype tested were infested with eggs obtained from a stock colony of CLM maintained on 'Cravo' lemon (*Citrus limonia* L. Osbeck), and the duration and survival of the eggs, larval and pupal stages, pupal size and weight, fecundity and longevity of adults, and sex ratio were evaluated. No influence was observed on the duration and survival of eggs, larvae and pupae of *P. citrella*. However, pupae obtained in the hybrid C x R<sub>4</sub> were significantly smaller and lighter than pupae from the remaining treatments. Adult females from the hybrids C x R<sub>4</sub> and C x R<sub>315</sub> were the least fecund. However, the lowest value for the corrected reproductive potential (CRP) was recorded in the hybrid C x R<sub>315</sub>, suggesting that this genotype is the least favorable for the development and reproduction of CLM. On the other hand, the highest CRP value obtained in the 'Rugoso' lemon confirms the susceptibility of this genotype, indicating it as the most suitable for CLM.

#### Introduction

The citrus leafminer (CLM), *Phyllocnistis citrella* Stainton, is considered one of the most important pests in citrus growing areas. Besides the direct damage (reduction in photosynthesis, leaf loss and constraint of new sprouts) (Heppner 1993), CLM also causes indirect damage by increasing the plant's susceptibility to pathogens such as the canker bacteria, *Xanthomonas axonopodis* pv. *citri* (Cook 1988).

Considering the negative aspects of traditional chemical control, the development of alternative methods to control CLM is necessary. Among the strategies

applied in integrated pest management programs, plant resistance to insects can be explored as a complementary control method. Investigations aiming to find new resistance factors in citrus varieties against insects have been conducted in several countries as India, USA, Spain and Argentina (Batra & Sandhu 1983, Peña & Ducan 1993, Garijo & Garcia 1994, Goane *et al* 2008), but are still very rare in Brazil, especially against CLM (Milano 2002). Considering the social and economical importance of the citrus agribusiness in Brazil, and the lack of information on the development and reproduction of CLM in different citrus cultivars available in the country, we aimed to identify genotypes carrying CLM resistant traits. The

identification of such genotypes would play a major role in setting the ground basis to include plant resistance in the integrated pest management of this insect.

## Material and Methods

### *Insect colony and plant genotypes*

Adults of *P. citrella* were obtained from a lab colony maintained on 'Cravo' lemon (*Citrus limonia*) under controlled laboratory conditions ( $25 \pm 1^\circ\text{C}$ ,  $70 \pm 10\%$  RH and 14h photophase), following Chagas & Parra (2000).

The citrus genotypes tested were 'Sunki' tangerine (*Citrus sunki* Hort.), the hybrids C x R<sub>4</sub> (*C. sunki* x *Poncirus trifoliata*), C x R<sub>315</sub> (*C. sunki* x *P. trifoliata*), M x P<sub>222</sub> [*Citrus sinensis* x Murcott tangor (*Citrus reticulata* x *C. sinensis*)] and 'Trifoliata Limeira' (*P. trifoliata*), which were previously selected as resistant to CLM (Santos 2009). The 'Rugoso' lemon (*Citrus jambhiri*) was used as control due to its known susceptibility. These genotypes were multiplied by grafting and grown in plastic bags (3.8 L) containing a mix of pinus husk and vermiculite. The transplants were maintained in a greenhouse, cut at 1/3 from the top and irrigated twice a week with a nutritive solution containing in mg.l<sup>-1</sup>: Ca(NO<sub>3</sub>)<sub>2</sub> = 58.2; Fe = 10.8; K<sub>2</sub>SO<sub>4</sub> = 5.4; KNO<sub>3</sub> = 40; MAP = 10.8; MgSO<sub>4</sub> = 16.4; (NH<sub>4</sub>)<sup>+</sup>(NO<sub>3</sub>)<sup>-</sup> = 1.4, ZnSO<sub>4</sub> = 2.73, according to IAC recommendations (Centro APTA Citros Sylvio Moreira).

To perform the tests, the transplants (approximately 50 cm high) were visually selected considering the number of leaves and the size of sprouts. Initially, plants were washed to eliminate any unwanted mites and/or insects.

To obtain CLM eggs, three couples were maintained in 100 ml polystyrene cages during 24h for adaptation and mating under controlled conditions ( $25 \pm 1^\circ\text{C}$ ,  $70 \pm 10\%$  RH and 14h photophase). Moistened filter paper discs were used to cover the cage walls, and after the adaptation period (24h), the adults were transferred to the new cages where they remained for 48h for egg laying, which was a sufficient time to obtain a large amount of eggs (Santos 2009). Adults were then removed and the eggs laid on the leaves were counted. Fifteen to forty eggs per plant were used in each treatment due to the natural variability in the number of eggs laid per plant.

Egg, larval and pupal developmental time and survival were recorded daily. On the 13<sup>th</sup> day after infestation, all leaves containing CLM pupae were removed from the plants and individually transferred to containers for adult emergence and further assessment of longevity.

The size and weight of pupae were assessed after artificial infestation of each tested genotype with three CLM couples for 72h, using five replicates/genotype. After oviposition ceased, adults were removed and plants were maintained under controlled conditions

for up to 13 days; CLM pupae were then removed from their pupal chambers built on the leaves, and their size and weight assessed after 24h. Pupae were separated and their size measured using a digital equipment (Wild model MMS 235) coupled to a stereomicroscope, while their weight was assessed using an analytical scale (0.1 mg precision).

Pupae from each genotype were isolated within cages, the emerged adults sorted by sex using a microscope and their survivorship was daily recorded in the presence and absence of a food source (honey). Thirty-five pupae per treatment and genotype were used, with each pupa considered as a replicate.

Fecundity and fertility were assessed on the adults obtained as previously described. Twenty couples were tested per genotype evaluated. One pair of couples was placed in a cage adapted from ethylene terephthalate (PET) bottles, in which a ventilation hole was made on one of the sides and covered with voile cloth. Adults obtained from all the tested genotypes were offered the same genotype ('Cravo' lemon) to assess their fecundity and survivorship. Adults were fed pure honey applied on the sides of the cage. Plants were replaced every other day. Each experiment had ten replicates, with each pair of CLM couples considered a replicate. The number of eggs laid was counted every time the plant was replaced, while egg survival was assessed on the 5<sup>th</sup> day.

We also used the Corrected Reproductive Potential (CRP) as proposed by Vendramim & Fancelli (1988) to verify the effects of each genotype on the development and reproduction of CLM, as well as to quantify their resistance level. The CRP was calculate as:  $CRP = (sr \times A)^n$ , where  $sr = \text{sex ratio} = \frac{\text{♀}}{\text{♀} + \text{♂}}$ , A = number of adults able to mate, n = number of generations during the period of CRP assessment.

All assays were conducted in randomized blocks. Data from all tests were submitted to Shapiro Wilk test to verify for normality, as well as to Bartlett's test to verify for homoscedasticity. If normal distribution was obtained or if the data were properly transformed, the data were subjected to ANOVA and to parametric tests to identify differences among treatments using a probability of 5%. Data that did not fit normal distribution were subjected to non-parametric tests. Analyses were performed using the statistical software R (version 2.7.2, 2008).

## Results and Discussion

The embryonic (F = 1.17; P = 0.35), larval (F = 1.21; P = 0.33) and pupal (F = 0.60; P = 0.70) development of CLM were not affected by the different genotypes tested (Table 1). The developmental time observed for eggs (4.2-4.5 days) and pupae (8.1-9.6 days) in the genotypes of tangerine were close to those obtained on different

Table 1 Development time and survival of the different stages of development of *Phyllocnistis citrella* reared on different citrus genotypes (mean  $\pm$  standard error) ( $25 \pm 1^\circ\text{C}$ ;  $70 \pm 10\%$  RH; 14h photophase).

Genotype	Duration (days)				Survival (%)			
	Egg <sup>ns</sup>	Larva <sup>ns</sup>	Pupa <sup>ns</sup>	Egg to adult <sup>ns</sup>	Egg <sup>ns</sup>	Larva <sup>ns</sup>	Pupa <sup>ns</sup>	Egg to adult
<i>P. trifoliata</i>	4.5 $\pm$ 0.19	7.1 $\pm$ 0.30	9.1 $\pm$ 0.84	20.8 $\pm$ 0.61	97.0 $\pm$ 1.84	87.5 $\pm$ 4.65	97.0 $\pm$ 3.00	82.7 $\pm$ 6.41 ab
C x R <sub>315</sub> hybrid	4.4 $\pm$ 0.09	7.3 $\pm$ 0.30	9.6 $\pm$ 0.39	21.4 $\pm$ 0.59	97.5 $\pm$ 5.23	90.1 $\pm$ 9.57	94.8 $\pm$ 2.18	82.8 $\pm$ 9.78 ab
M x P <sub>222</sub> hybrid	4.4 $\pm$ 0.16	6.9 $\pm$ 0.55	9.0 $\pm$ 0.59	20.3 $\pm$ 0.66	89.3 $\pm$ 2.40	84.0 $\pm$ 3.02	96.9 $\pm$ 3.89	72.3 $\pm$ 2.63 b
C x R <sub>4</sub> hybrid	4.3 $\pm$ 0.09	8.3 $\pm$ 0.40	8.1 $\pm$ 0.41	20.6 $\pm$ 0.48	100.0 $\pm$ 1.20	92.0 $\pm$ 4.19	92.9 $\pm$ 1.56	85.1 $\pm$ 4.42 ab
'Sunki' tangerine	4.3 $\pm$ 0.07	7.5 $\pm$ 0.50	8.9 $\pm$ 0.72	20.6 $\pm$ 0.64	98.8 $\pm$ 0.00	91.6 $\pm$ 4.15	96.3 $\pm$ 2.99	86.8 $\pm$ 4.56 ab
'Rugoso' lemon	4.2 $\pm$ 0.06	7.1 $\pm$ 0.55	8.8 $\pm$ 0.87	20.0 $\pm$ 0.65	98.2 $\pm$ 1.11	98.5 $\pm$ 0.87	100.0 $\pm$ 0.00	96.8 $\pm$ 1.64 a
CV (%)	6.14	13.56	16.69	6.57	5.90	12.65	6.02	14.77

Means followed by the same letter in columns are not significantly different ( $P > 0.05$ , Tukey's test); ns = Non significant.

cultivars of lime and acid lime under semi controlled conditions (Patel & Patel 2001) and on citrus cultivars under field conditions (Wilson 1991). However, egg development on 'Cravo' lemon was half of that observed on tangerine (Chagas & Parra 2000). Larval development was shorter on tangerine (8.0-9.5 days) than on lime and acid lime (8.6-10.6 days) (Patel & Patel 2001), but around 2-3 days longer than in 'Cravo' lemon (Chagas & Parra 2000).

No significant differences were observed for the survival of eggs ( $F = 2.28$ ;  $P = 0.08$ ), larvae ( $F = 0.91$ ;  $P = 0.49$ ) and pupae ( $F = 0.88$ ;  $P = 0.51$ ) (Table 1). These values were much higher than those obtained on lime and acid lime under semi controlled conditions (Patel & Patel 2001) or under field conditions on several citrus cultivars (Wilson 1991), but very similar to those reported on 'Cravo' lemon (Chagas & Parra 2000).

While most of the larval mortality observed occurred in the first instar, genotypes *P. trifoliata* and C x R<sub>4</sub> hybrid also had some larval mortality on day 13, when larvae in most of the genotypes had already initiated the pupal stage. The lower survival observed for the immature stage on the M x P<sub>222</sub> hybrid as compared to the 'Rugoso' lemon is not necessarily an indicative of plant resistance, as it may be related to the abortion of infested leaves, which limited the evaluation of CLM development on this hybrid. Leafminer infestations are known to stimulate plants to produce and accumulate ethylene in the petioles of their leaves inducing leaf abscission as a possible strategy of plants to resist the attacks of these pests (Risley 1986, Kappel *et al* 1987, von Dahl & Baldwin 2007).

The size of the pupae was highly affected by the genotype where the insects developed on ( $F = 13.52$ ;  $P < 0.001$ ) (Table 2). The smallest (1.7 mm) pupae were observed on the C x R<sub>4</sub> hybrid, although not different from those obtained from the C x R<sub>315</sub> genotype (1.9 mm). Except for these two genotypes, the size of the pupae was in the range reported for several other citrus varieties

Table 2 Pupal size (mm) and weight (mg) of *Phyllocnistis citrella* reared on different citrus genotypes (mean  $\pm$  standard error) ( $25 \pm 1^\circ\text{C}$ ;  $70 \pm 10\%$  RH; 14h photophase).

Genotype	Size (mm)	Weight (mg)
C x R <sub>4</sub> hybrid	1.7 $\pm$ 0.06 d	0.1 $\pm$ 0.02 b
C x R <sub>315</sub> hybrid	1.9 $\pm$ 0.07 cd	0.2 $\pm$ 0.03 ab
'Rugoso' lemon	2.0 $\pm$ 0.02 bc	0.2 $\pm$ 0.02 ab
<i>P. trifoliata</i>	2.1 $\pm$ 0.07 bc	0.2 $\pm$ 0.02 ab
'Sunki' tangerine	2.2 $\pm$ 0.09 ab	0.4 $\pm$ 0.19 a
M x P <sub>222</sub> hybrid	2.4 $\pm$ 0.05 a	0.3 $\pm$ 0.04 ab
CV (%)	8.90	40.65

Means followed by the same letter in columns are not significantly different ( $P > 0.05$ , Tukey's test); original data were transformed in  $\sqrt{x}$  for statistical analysis.

(2.05-2.79 mm) (Jacas *et al* 1997). However the size of the pupae from all tangerine genotypes and 'Rugoso' lemon was much smaller than the size reported on oranges, lemon and pommel (2.59-2.77 mm) (Goane *et al* 2008). Pupal weight was not affected by the genotype tested ( $F = 2.17$ ;  $P = 0.09$ ) (Table 2), suggesting that this parameter was not significant to identify citrus resistance sources to CLM. Although not common in CLM studies, pupal weight is a parameter that may help to distinguish the effects of different genotypes on CLM development.

No differences were observed on the sex ratio of insects reared on the genotypes tested ( $F = 0.58$ ;  $P = 0.72$ ) (Table 3). The sex ratio was close to 0.5, similar to what has been reported for CLM in several studies (Raga *et al* 1998, Parra *et al* 2002), but lower than the sex ratio reported for insects reared on lemon, orange and pommel (nearly 0.7) (Goane *et al* 2008).

Differences in longevity among adults reared on different citrus genotypes were observed only for females in the presence of food ( $F = 6.33$ ;  $P < 0.001$ ), while no

Table 3 Sex ratio, longevity of adults, fecundity and fertility of *Phyllocnistis citrella* reared on different citrus genotypes (mean  $\pm$  standard error) ( $25 \pm 1^\circ\text{C}$ ;  $70 \pm 10\%$  RH; 14h photophase).

Genotype	Sex ratio <sup>1, ns</sup>	Longevity (days) <sup>1</sup>				Fecundity <sup>2</sup> (eggs/ female)	Fertility <sup>1</sup> (%)
		Female		Male			
		fed	unfed	fed <sup>ns</sup>	unfed		
C x R <sub>4</sub> hybrid	0.4 $\pm$ 0.04	14.5 $\pm$ 1.26 b	2.2 $\pm$ 0.21 b	14.7 $\pm$ 2.50	2.1 $\pm$ 0.14 ab	92.1 $\pm$ 10.20 b	98.0 $\pm$ 0.62 ab
C x R <sub>315</sub> hybrid	0.5 $\pm$ 0.06	16.5 $\pm$ 2.18 b	2.5 $\pm$ 0.18 ab	17.6 $\pm$ 2.98	2.6 $\pm$ 0.26 a	94.6 $\pm$ 12.31 b	94.0 $\pm$ 1.08 b
M x P <sub>222</sub> hybrid	0.5 $\pm$ 0.06	15.9 $\pm$ 2.13 b	3.1 $\pm$ 0.11 a	18.0 $\pm$ 1.70	2.6 $\pm$ 0.18 a	125.9 $\pm$ 11.02 ab	99.0 $\pm$ 0.34 a
'Sunki' tangerine	0.5 $\pm$ 0.07	11.6 $\pm$ 2.32 b	2.7 $\pm$ 0.14 ab	17.8 $\pm$ 2.94	2.3 $\pm$ 0.14 ab	116.5 $\pm$ 10.77 ab	99.0 $\pm$ 0.47 a
'Rugoso' lemon	0.5 $\pm$ 0.03	25.5 $\pm$ 1.87 a	2.4 $\pm$ 0.23 ab	16.9 $\pm$ 2.49	1.8 $\pm$ 0.15 b	153.0 $\pm$ 12.06 a	99.0 $\pm$ 0.28 a
<i>P. trifoliata</i>	0.5 $\pm$ 0.10	18.1 $\pm$ 1.84 b	2.8 $\pm$ 0.23 ab	14.9 $\pm$ 0.89	2.3 $\pm$ 0.18 ab	113.7 $\pm$ 5.30 ab	96.0 $\pm$ 0.90 b
CV (%)	26.35	41.00	26.80	57.06	33.00	-----	2.21

<sup>1</sup>Means followed by the same letter in columns are not significantly different ( $P > 0.05$ , Tukey's test); <sup>2</sup>Means followed by the same letter in columns are not significantly different ( $P > 0.05$ , Kruskal Wallis test); ns = non significant.

differences were observed for fed males ( $F = 0.44$ ;  $P = 0.82$ ) (Table 3). Longevity values reported here are higher than those reported by Chagas & Parra (2000). These discrepancies might arise from the different genotypes from which they were reared in these studies.

Longevity of unfed males ( $F = 3.50$ ;  $P = 0.005$ ) and females ( $F = 2.87$ ;  $P = 0.01$ ) was also affected by the tested genotypes (Table 3). Males from 'Rugoso' lemon were short-lived (1.8 days) as compared to males from C x R<sub>315</sub> and M x P<sub>222</sub> hybrids (2.6 days), while females from M x P<sub>222</sub> hybrid lived longer than females from the C x R<sub>4</sub> hybrid (Table 3). Considering that there is a great variation in longevity among fed and unfed individuals, both situations should be considered when evaluating variety resistance.

Fecundity was also affected by the genotypes tested ( $X^2 = 17.48$ ;  $P = 0.003$ ) (Table 3). Fecundity of fed females obtained from C x R<sub>4</sub> and C x R<sub>315</sub> hybrids were much lower than of those reared on 'Rugoso' lemon, while all other genotypes yielded females with intermediate fecundities (Table 3). The CLM fecundities, even on the least suitable genotype, were higher than those reported on 'Cravo' lemon (Chagas 1999). The 'Cravo' lemon cultivar is known to be susceptible to CLM, and a higher fecundity would be expected compared to the tangerine genotypes we tested, but different physiological and genetic traits between the two populations studied could explain such differences.

Egg survival was also influenced by the genotype tested ( $F = 9.20$ ;  $P < 0.01$ ) (Table 3); eggs laid by females reared on C x R<sub>315</sub> hybrid and *P. trifoliata* had much lower survival than the eggs of females from the remaining genotypes, except the C x R<sub>4</sub> hybrid (Table 3).

The results on the reproductive capacity of CLM on the studied genotypes indicate the C x R<sub>315</sub> hybrid as the one that most interfered on fecundity and fertility, revealing

its potential as a source material for citrus improvement programs against CLM.

Fecundity varied as females aged and egg numbers on these genotypes ranged from 0.2 to 27.1 eggs/two days (Fig 1). Oviposition peaked between the 4<sup>th</sup> and the 12<sup>th</sup> day for all genotypes. After the 18<sup>th</sup> day, there was a significant reduction in the number of eggs laid for all tested genotypes.

Females reared from 'Rugoso' lemon actively laid eggs up to the 28<sup>th</sup> day, while those reared from M x P<sub>222</sub> hybrid, 'Sunki' tangerine and on *P. trifoliata* had a 2-d shorter oviposition period, and those from C x R<sub>315</sub> and C x R<sub>4</sub> hybrids had a 4-d shorter oviposition period (Fig 1).

Thus, when all parameters are individually taken into account, the survival of the egg-adult period, the pupal size and weight, and longevity and fecundity were the best biological parameters to characterize the genotype influence on CLM development. An analysis including all CLM biological parameters observed in here on the different genotypes yielded CRP values that also indicate the C x R<sub>315</sub> and C x R<sub>4</sub> hybrids as the least favorable to CLM development and reproduction (Table 4), which would be expected to present the lowest rates of CLM population increase over time.

Understanding the interactions between the insects and the plants is an important tool for the development of integrated management strategies of leafminers in commercial citrus orchards, especially due to the increased social demand for alternative pest control techniques. According to our results, C x R<sub>315</sub> and C x R<sub>4</sub> hybrids contain resistance factors against CLM. C x R<sub>315</sub> and C x R<sub>4</sub> hybrids are genotypes that originated from crossings between *C. sunki* x *P. trifoliata* cv. Rubidoux. Since *P. trifoliata* is known to carry a gene that leads to an antibiosis response to CLM (Berne *et al* 2005), and *C. sunki* is known to be susceptible to CLM, it is likely



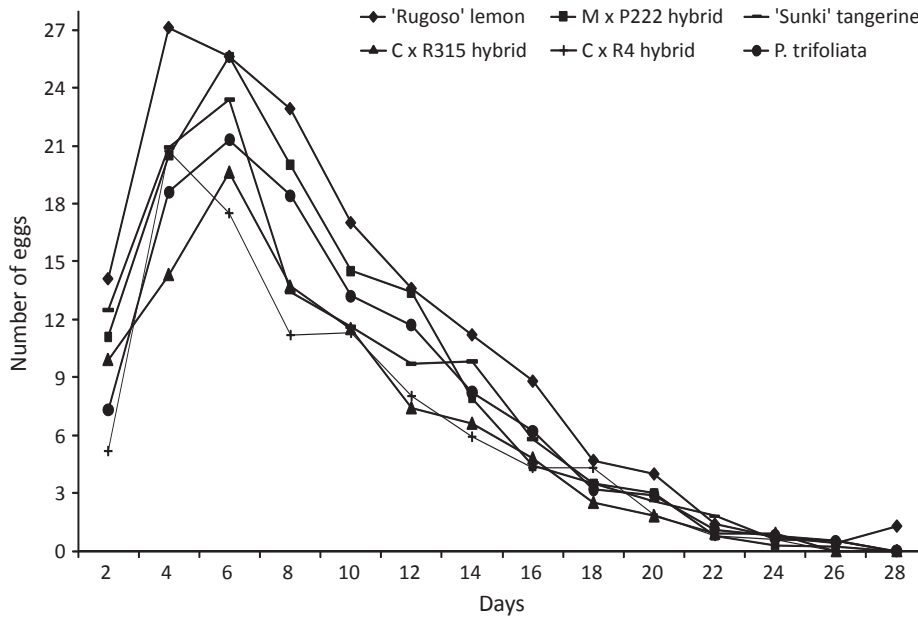


Fig 1 Bi-daily rhythm of egg laying of *Phyllocnistis citrella* reared on different citrus genotypes ( $25 \pm 1^\circ\text{C}$ ;  $70 \pm 10\%$  RH; 14h photophase).

Table 4 Corrected reproductive potential (CRP) of *Phyllocnistis citrella* on different citrus genotypes for a period of 60 days ( $25 \pm 1^\circ\text{C}$ ;  $70 \pm 10\%$  RH; 14h photophase).

Genotype	CRP	
	Absolute value	Relative value
C x R <sub>315</sub> hybrid	9.937	1
C x R <sub>4</sub> hybrid	11.067	1.1
<i>P. trifoliata</i>	14.730	1.5
'Sunki' tangerine	23.048	2.3
M x P <sub>222</sub> hybrid	26.698	2.7
'Rugoso' lemon	92.539	9.3

that the resistance traits observed in C x R<sub>315</sub> and C x R<sub>4</sub> hybrids may be linked to the resistance trait observed in *P. trifoliata*. It is also likely that the genetic resistance mechanism observed in *P. trifoliata* may be a characteristic of the genus *Poncirus*. These results are relevant to the selection of new materials for further research into the genetic improvement of citrus cultivars to CLM, and these genotypes deserves further investigation.

### Acknowledgments

To Dr. Marcos A. Machado and Dr. Mariângela Cristofani-Yali (Centro APTA Citros Sylvio Moreira) for supplying the different citrus genotypes tested and for their suggestions, to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarships (process n° 140462/2005-8), and to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant number 2006/56434-5) for funding this project.

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