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Evaluation of sampling methods of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) in two citrus growing areas

Evaluación de métodos de muestreo de *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) en dos áreas citrícolas

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ABSTRACT

Efficient, easy to implement and low-cost monitoring methodologies are necessary to obtain information on arthropod pest populations and to implement the most convenient and timely phytosanitary control practices. To optimize the sampling and monitoring of the Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), an insect associated with the transmission of *Candidatus* Liberibacter asiaticus and *Ca.* L. americanus, which cause Huanglongbing (HLB), a disease with a great impact on citrus orchards, three methods of adult sampling (yellow sticky traps, sweep net, and stem tap) were evaluated. For immature sampling, vegetative shoots were checked. The results demonstrate that the population density at the time of sampling affects the effectiveness and sensitivity of the sampling methods. Yellow sticky traps capture more adults and are the only effective method at low psyllid densities. Stem tap and sweep net are less expensive methods; however, they do not detect adults nor correlate with the number of nymphs and eggs in vegetative shoots when adult density is low. For adults, an optimal sample size was determined for each method. For yellow sticky traps, 3 to 5 traps for a 2-hectare plot with weekly frequency are recommended. For immatures, it is recommended to estimate the percentage of infestation by inspecting 45 to 55 vegetative shoots well-distributed within a 2-hectare plot, as a practical measure for farmers and extensionists to monitor *D. citri*.

Keywords: Huanglongbing; Integrated pest management; Monitoring; Sticky traps; Vectors

RESUMEN

Metodologías de monitoreo eficientes, fáciles de implementar y de bajo costo, son necesarias para obtener información sobre las poblaciones de artrópodos plaga e implementar las prácticas de control fitosanitario más convenientes y oportunas. Con el fin de optimizar el monitoreo del psílido asiático de los cítricos *Diaphorina citri*, vector de Huanglongbing, enfermedad con gran impacto en plantaciones de cítricos, se evaluaron tres métodos de muestreo de adultos: trampas pegajosas amarillas, pase de jama y golpeteo o golpe de rama. Para el muestreo de inmaduros se inspeccionaron brotes vegetativos. La densidad poblacional en el momento del muestreo afecta la efectividad y sensibilidad de los métodos de muestreo. Las trampas pegajosas amarillas tienen más capturas de adultos y por su sensibilidad es el único método conveniente con baja densidad del psílido. La adopción de los métodos de golpe de rama y pase de jama es más económica; sin embargo, no detectan adultos, ni se correlacionan con el número de ninfas y huevos en brotes vegetativos, cuando la densidad de adultos es baja. Para adultos, se determinó un tamaño de muestra óptimo para cada método de muestreo. Se recomiendan monitoreos de adultos semanales con 3 a 5 trampas amarillas pegajosas en un lote de dos hectáreas (has). Para inmaduros se recomienda estimar el porcentaje de infestación a partir de la inspección de 45 a 55 brotes vegetativos bien distribuidos dentro de un lote de dos has, como una medida práctica para monitorear *D. citri*, por parte de agricultores y extensionistas.

Palabras clave: Huanglongbing; Manejo integrado de plagas; Monitoreo; Trampa pegajosa; Vector.

INTRODUCTION

Huanglongbing (HLB) or citrus greening is a disease caused by the bacterium *Candidatus* Liberibacter asiaticus, which restrict to the phloem, reduces the quality and quantity of production, and subsequently causes plant death (Dala-Paula *et al.* 2019; Achor *et al.* 2020). It has already been detected in citrus crops in Asia, Africa, and America (Parnell *et al.* 2019). The social, environmental, and economic impact has been significant in the main production centers of the world where it has become established (Costa *et al.* 2021).

In Colombia, this disease was reported in 2015, nevertheless, its vector, *Diaphorina citri* (Hemiptera: Liviidae), is present in much of the national territory, the disease remains primarily restricted to the Caribbean region (ICA, 2017) and Santander (ICA, 2023). However, the potential distribution of HLB and its vector, *D. citri*, suggests that most of the Colombian citriculture is at risk of contracting the disease (Olvera-Vargas *et al.* 2020).

Within integrated vector management, in areas with and without HLB, it is essential to have efficient, sensitive, easy-to-adopt, and low-cost sampling methodologies (Monzo *et al.* 2015). Monitoring and the use of action thresholds for *D. citri* control are necessary because they have the potential to reduce insecticide applications, mainly in areas with the disease, where calendar-based applications are made (Monzo & Stansly, 2017). Several studies have evaluated sampling techniques typically used for capturing hemipterans and have highlighted their disadvantages and advantages for *D. citri* sampling (Hall, 2009; Stansly *et al.* 2010). In some cases, the most effective methods for capturing adults have been identified, and in others, the optimal number of samples has been determined (Hall *et al.* 2007; Sétamou *et al.* 2008; Monzo *et al.* 2015; Leong *et al.* 2019).

Under current Colombian regulations, all citrus orchard owners are required to monitor for *D. citri* and control it if necessary. The same applies to live fences of myrtle (*Murraya paniculata*) or limon swinglea (*Swinglea glutinosa*), which are alternate host plants for the vector and the disease (Santos *et al.* 2020). Farmers and extensionists use monitoring strategies based on research generated in subtropical regions and countries with environmental conditions different from those in Colombia. Ideally, monitoring methodologies should be validated for each region (Varón-Devia *et al.* 2020).

Although the sampling methodologies proposed for other countries are low-cost, it is not known how sensitive and reliable they can be in the citrus-growing areas of Colombia. The best method, in addition to regional variability, could depend on the sampling objectives. Sampling in areas with HLB, where the incidence should be zero, is different from areas without HLB, where the objective is to reduce populations (Varón-Devia *et al.* 2020).

Therefore, different methods for monitoring immatures and adults of *D. citri* were compared to generate an efficient sampling strategy in two citrus growing areas of Colombia: The Caribbean coast and the department of Santander in the Andean region.

MATERIALS AND METHODS

Study area. The study was conducted in the Andean region in the municipality of Girón, Santander, a region without the presence of the disease during the experiment (Figure 1). In a four-year-old organic Tahiti acid lime orchard located at coordinates 6°59'42.5"N; 73°10'24.2"W, at an altitude of 809 meters above sea level, sampling was carried out biweekly from December 2019 to November 2020.

On the Colombian Caribbean coast, sampling was conducted in a Tahiti acid lime orchard with the presence of the disease. The orchard is in the municipality of Dibulla, La Guajira, a department affected by HLB since 2015, with coordinates N 11°14'18.36"; W 73°32'2.43" (Figure 1). Biweekly sampling was conducted from February to November. HLB is present in this orchard, as in the entire department.

Sampling in citrus orchards. A 2-hectare planting area was delimited and divided into 10 individual 0.2-hectare plots in the two locations. Four trees were selected per plot for 40 trees in total. In all samplings, the same trees were evaluated using the following capture methodologies:

<u>Yellow Sticky trap:</u> Letter-size (23x30 cm) 520 - 615 nm traps were used, suspended 1.5 m above the ground near the outside of a branch canopy. The traps were changed every 15 days, and two traps were placed on opposite sides of the tree.

In Girón samplings, in 19 of the 40 trees, the traps were placed in the north and south orientation of the tree, while in the remaining ones, it was east and west. In the Dibulla samplings, all pairs of traps per tree were in the north and south orientation. The arrangement of the traps and the difference between the two regions was because in the Caribbean region it was known from previous studies the predominance of north-south winds, since the wind direction is correlated with the dispersion of *D. citri* (Johnston *et al.* 2019), the location of the traps in the north-south orientation was preferred. While in Santander the predominant wind direction was unknown, and the four cardinal points were evaluated. To count the adults of *D. citri*, the traps were placed in properly labeled plastic bags and then carefully checked using a stereomicroscope.

<u>Stem-tap:</u> A branch was randomly selected, which must be 1.5 m high and outside the tree canopy. This branch was repeatedly tapped three times with a 0.5-inch diameter PVC pipe, while a rectangular plastic tray, preferably white, was placed under the branch. In this way, all adult psyllids that fell from the branch to the tray were quickly counted before they escaped.

<u>Sweep net:</u> Twice per tree, three double passes were made with the sweep net, making sure to pass through the buds and branches of the tree, but without entanglement. Subsequently, the interior of the net was observed to count the total number of adults captured. In Girón, this methodology was applied on 10 of the 14 sampling dates.

In addition, four shoots were randomly collected per tree in Girón. While in Dibulla two were collected, one in the north orientation of the tree canopy and the other in the south. These shoots were placed

Figure 1. Departments and municipalities in Colombia of capture of adults of the Asian citrus psyllid *Diaphorina citri* with three sampling methods.

in paper bags in coolers with refrigerant gel to prevent them from drying out. The number of *D. citri* eggs and nymphs in each shoot was carefully examined and counted using a stereomicroscope.

Statistical analysis. To compare the abundance and incidence of *D. citri* adults detected by the sampling methods: yellow sticky traps, stem-tap, and sweep net, a Friedman test was performed, which is a non-parametric alternative when paired data are available.

For abundance, the two replicates of each sampling methodology were added to have a single value per tree and per sampling date. In the case of incidence, the percentage of trees with *D. citri* from each sampling date was compared between sampling methodology, also with a paired analysis of the data. Comparisons were made separately for each location.

Nested analyses of variance were performed for each sampling method, with trees nested in each plot, nested in sampling dates. The count data for this analysis will be log transformed [log (x+1)].

The analysis of variance for egg and nymph counts on shoots was carried out with the information from the sampling dates with the highest sprouting when all 40 trees in the trial had shoots.

The test to define the optimal number of samples (Sétamou *et al.* 2008; Leong *et al.* 2019), was performed. This analysis considers that there are two levels or sampling factors, the variance between trees and between sampling units within the same tree. The optimum number of trees to be sampled is obtained using the following equation.

$$t = \frac{\left(\delta t^2 + \frac{\delta u^2}{u}\right)}{(\bar{x} + E)^2}$$

The variance components between trees and between sampling units within the same tree were estimated using the statistical program StatGraphics Centurion version XV.

On the other hand, "*u*", refers to the number of sampling units such as traps, stem tap or net passes within the same tree; "*E*", refers to the deviation with respect to the mean, we worked with 25% and 10% (0.25 and 0.1 in the equation respectively). For pest management decisions, estimates with a deviation of 25% from the mean are considered acceptable (Southwood & Henderson, 2000).

To evaluate the relationship between adults and immatures, a correlation matrix was performed between the count of eggs and nymphs in shoot and the count of adults for each adult sampling methodology, a non-parametric Spearman rank correlation coefficient (ρ) was estimated with the PAST version 1.86b program. Sampling points without flush shoots due to the impossibility of recording immatures of *D. citri,* as well as sampling points with captures of adults ≤2 individuals in the entire evaluated period, were not included. To evaluate the differences between quadrants, East-West and Nort–South, the Wilcoxon signed-rank test was performed, which is a nonparametric paired analysis.

In addition, a correlation was made between egg and nymph counts and percent shoot infestation in each 0.2 ha plot to assess the possibility that percent infestation, which is an easier measure to gather in the field, could replace the more cumbersome and inaccurate nymph and egg counts.

To determine the optimal number of trees to sample for the percentage of shoot infestation, the proposal in Southwood & Henderson (2000) was used to sample the frequency of occurrence of an event with the following equation:

$$N = \frac{t^2 p(1-p)}{D^2}$$

Where "*N*" is the number of shoots to be sampled; "*p*" is the preliminary value of the probability of the event, in this case, the average infestation percentage of all samplings, "*D*" is as half the width of the confidence limit regarding the estimate of the mean in decimal, "*t*" is the value of the student t distribution, which depends on the number of samples and is approximated to 2 with more than 20 samples.

RESULTS AND DISCUSSION

Evaluation of adult sampling methods. Significant differences were found between the adult sampling methods (Figure 2). Yellow traps captured more adults, even at sampling points that did not record adults with the sweep net and stem tap, yellow traps reached mean values of 7.44 adults/tree in Girón and 0.65 adults/tree in Dibulla. Likewise, the sweep net method in Dibulla presented 2 captures of adults in the entire sampling period. Between the sweep net and stem tap methods differences were present only in Girón (Figure 2). Studies demonstrated that yellow traps were the most efficient sampling method at low densities because they have 14 times more sensitivity in capturing adults (Monzo *et al.* 2015; Miranda *et al.* 2017). Yellow traps were also considered the best option in areas with the presence of HLB, with intensive management with pesticides, where it is more important to detect the presence than to estimate its population (Miranda *et al.* 2017; Varón-Devia *et al.* 2020). Monzo *et al.* (2015) indicate that the yellow trap method was more sensitive and convenient in periods with little or no sprouting and low densities of adults, as well as during the dry season or the phenological stage of fruit filling as recommended by Varón-Devia *et al.* (2020).

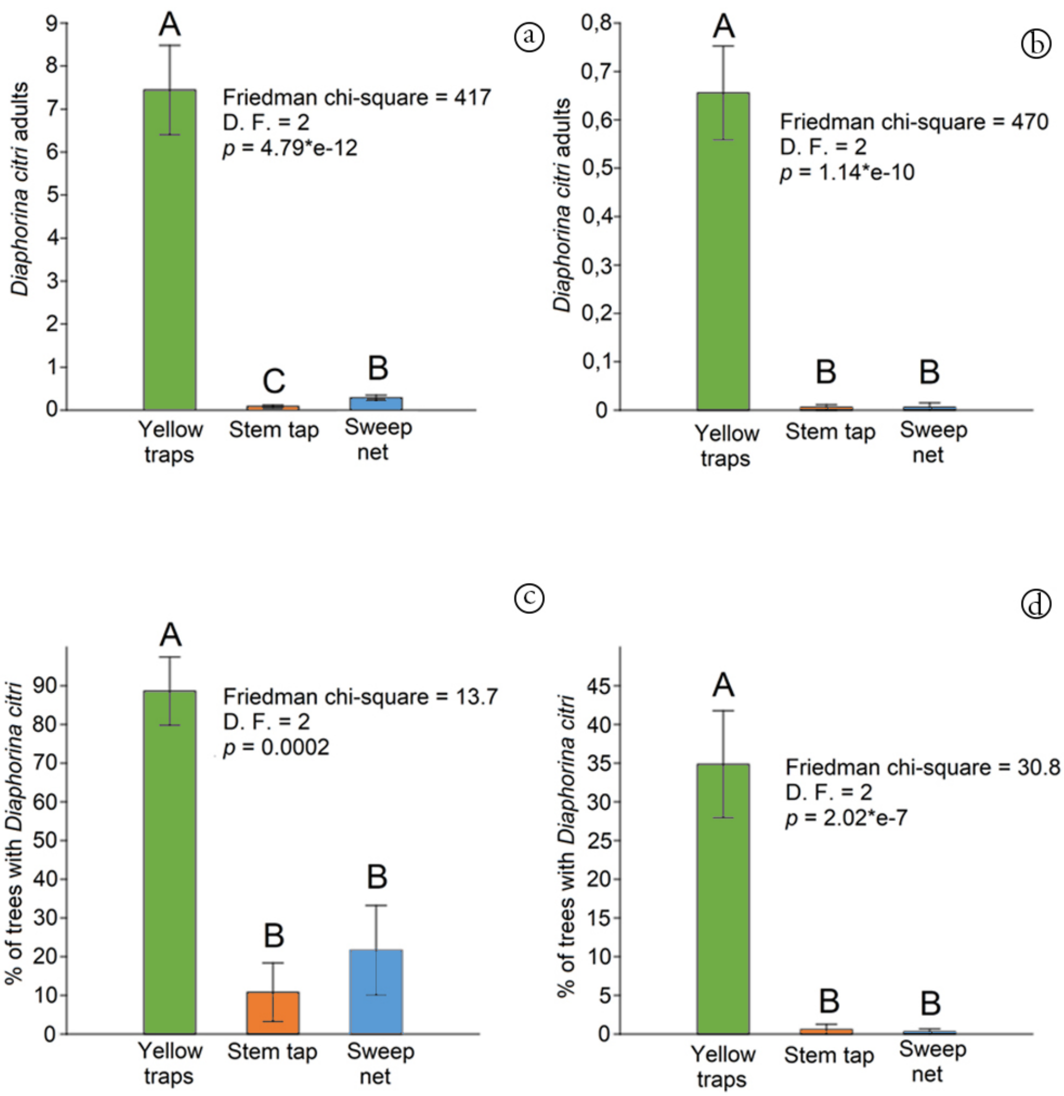

Figure 2. Capture of adults of the Asian citrus psyllid *Diaphorina citri* with three sampling methods. Abundance: a. Girón - Andean region; b. Dibulla - Caribbean coast. Presence – Absence: d. Girón - Andean region; e. Dibulla - Caribbean coast.
Comparison tests between treatments with Wilcoxon rank sum test with Bonferroni correction, different letters indicate significant differences.

The density of adults of *D. citri* in the crops monitored in this research was 3.78 and 0.33 adults per biweekly trap in Girón and Dibulla, respectively, with a record for these same localities of 6.4% and 0.3% incidence of *D. citri* by the stem-tap method. Clearly, the density of adults at the time of sampling can influence the choice of the most convenient method. Hall *et al*. (2007) found an average density of 14.8 adults per yellow trap per week. At this density, the tapping method is efficient and registers adults in 80% of the trees where the yellow traps also reported them, Therefore, they recommend the stem tap method because it has a similar or greater efficiency than yellow traps with less investment of resources and time. Monzo *et al*. (2015) also found that the stem tap method was the most efficient with medium and high densities of *D. citri* adults, delimited as more than 0.1 adults per stem tap.

Yellow sticky traps have been used extensively to understand key aspects of *D. citri* biology, such as distribution and seasonal variation, and to make management decisions at regional and farm level (Miranda *et al.* 2017; Díaz-Padilla *et al.* 2021; Álvarez-Ramos *et al.* 2022). However, for an adequate interpretation of the sampling data with yellow sticky traps, it is necessary to consider several aspects. First, the method evaluates the presence of adults during a period and not at a specific time. Therefore, sudden changes in populations, due to factors such as pesticide applications, climatic events, or other factors could underestimate or overestimate the adult populations. On the other hand, because the *D. citri* life cycle at 27°C is 14±1.2 days (Botero *et al.* 2014), a weekly monitoring frequency is recommended to improve the estimation of adults. It is also necessary to consider that yellow traps show correlation with climatic variables such as temperature, light, and wind direction, which can affect estimates of *D. citri* populations with respect to the time of sampling and tree canopy location (Hall, 2009; Johnston *et al*. 2019).

The captures of *D. citri* for the East-West ($P=0.018$; $W=9888$; $n=273$) and North-South ($P<0.0001$; $W= 10800$; $n=247$) quadrants showed significant differences in the Girón crop, with a greater number of individuals in the east and south. Although environmental factors that affect the dispersal of *D. citri*, such as wind speed and direction (Antolínez *et al.* 2022), were not measured, the greater capture in the south can be more related to the proximity to an abandoned citrus orchard, without pest management, and with high incidence of *D. citri.* In Dibulla, no difference was found between north and south orientation ($P<0.73$; $W=0.000115$; $n=680$).

Optimal sample size. In all methods, highly significant differences were found between sampling dates, demonstrating that *D. citri* populations are affected over time by sprouting periods. In Giron, for plots and trees within plots, differences were detected in the capture of adults by the yellow trap method and in the presence of nymphs and eggs on shoots (Table 1).

In Dibulla, the results are the same as in Girón for the sticky traps, but there are no differences in the sampling by stem tap and sweep net, influenced by the low number of individuals collected by these methods.

The optimum sample size for the yellow traps is in accordance with the recommendations of other authors (Table 2), and for plots of 2 ha, it would be between 3 and 5. While the sample size for stem tap method and sweep net recommendations in the bibliography are higher (Table 2).

These authors do not perform a statistical analysis to support these recommendations. However, more samples allow a better estimation, and the optimal number of samples is variable over time with factors such as the population mean (Monzo *et al*. 2015). The proposed values (Table 2) should be understood as a reference and if resources are available, it is recommended to increase the precision and take a greater number of samples.

Correlation between adults sampling and immatures. Sampling with yellow traps shows a correlation with the presence of nymphs and eggs in shoots in the two locations, while the correlation with stem tap was significant only in Girón Santander (Table 3).

The sweep net method was not correlated with *D. citri* immatures at either location. The data suggest that as the density of *D. citri* populations increases, the correlation between sampling methods for adults increases. And the correlation between these and the number of immatures in shoots also increases.

Quantifying the number of eggs and nymphs in the field is time-consuming and inaccurate. However, a relationship has been found between the percentage of shoot infestation and the number of eggs and nymphs of *D. citri* (Sétamou *et al.* 2008; Stansly *et al.* 2010), Therefore, the percent infestation could be used as a surrogate measure for the number of immatures in shoots if there is sufficient correlation between the two variables. In Girón Santander, the percentage of infestation was correlated with the number of nymphs in shoots ($\rho = 0.86$; $p= 1.16e-24$) and eggs ($\rho= 0.64$; $p=8.9e-11$).

Few farmers monitor *D. citri* population; only those who export do so because they must comply with good agricultural practices (GAP). They measure the percentage of immature infestation in shoots and use thresholds of 10 to 15% for pest management decisions. This threshold, although arbitrary, is stricter or more conservative than the one proposed by Monzo & Stansly (2017), of 0.2 average adults per stem tap, which was only reached at one sampling time (0.25 in Girón), while 15% or more of shoot infestation was reached in almost all sampling dates. It is important to complement the sampling of the percentage of immature infestation in shoots with an estimation of flush shoot density (Stansly *et al.* 2010).

Resources invested in sampling implementation. Considering materials and labor time, the implementation costs for the stem-tap method are estimated at $25,250 ($ = Colombian pesos) for 2 ha plot, and for the sweep net method at $110,250, for only one sampling. However, all materials can be reused, and considering that the evaluation is performed in one hour per week, the costs are approximately $344,000 annually for the stem-tap method and $429,000 for the sweep net method. For the yellow sticky trap method, the cost of a single sampling with two yellow traps is estimated to be $63,250. If the yellow stick traps are not reused, $635,000 would be spent per year. In addition, training is required for the identification of *D. citri* adults in the trap, a task that requires at least a five-fold magnifying glass (5X). Finally, the visual inspection method for immatures costs 52,250 per sampling and 371,000 per year.

Table 1. Nested analysis of variance for *Diaphorina citri* sampling methods.

Source of variation	Girón, Santander		Dibulla, Guajira	
	D. F.	M. S.	D. F.	M. S.
YELLOW STICKY TRAPS				
Date sampling	12	5,88**	16	0,184**
Plot (0.2 ha)	117	0,21**	153	0,035**
Tree	390	0,14**	510	0,031**
STEM TAP				
Date sampling	13	0,027**	14	0,00039
Plot (0.2 ha)	126	0,003	135	0,00045**
Tree	420	0,004*	450	0,00025
SWEEP NET				
Date sampling	9	0,0954**	16	0,000316
Plot (0.2 ha)	90	0,0154	153	0,000333
Tree	300	0,0111	510	0,000333
EGGS ON FLUSH SHOOTS				
Date sampling	7	0,215**	6	0,283
Plot (0.2 ha)	72	0,159**	42	0,099
Tree	240	0,118**	49	0,211**
NYMPHS ON FLUSH SHOOTS				
Date sampling	7	2,304**	6	0,299**
Plot (0.2 ha)	72	0,267**	42	0,140*
Tree	240	0,194**	49	0,134**

D. F. = Degrees of freedom; M. S. = Mean Square. * $P< 0,05$; ** $P<0,01$.

Table 2. The Optimal sample size for sampling methods for the citrus Asian psyllid *Diaphorina citri* in a 2-ha citrus orchard.

Optimal sample size for sampling methods	Deviation from the mean				Other references				
	0,25		0,1		Trees (Flushes/tree)				
ADULTS Trees to sample	G	D	G	D	Bouvet & Hochmaier (2020)	Stansly *et al.* (2010)	Monzo *et al.* (2015)	Sétamou *et al.* (2008)	Leong *et al.* (2019)
Yellow traps (two per tree)	2	4	7	16					
Yellow traps (one por tree)	3	5	8	20			22		
Stem tap (two per tree)	7	6	40	40	80				
Stem tap (one per tree)	9	9	52	55		10	75		
Sweep net (two per tree)	6	12	32	75					
Sweep net (one per tree)	9	16	45	100			36		
IMMATURE Trees to sample	G	D	G	D					
Eggs (four flushes per tree)	15	11	67	41	40 (2)			3 (8)	4 (6)
Nymphs (four flushes per tree)	12	7	46	28	40 (2)			10 (6)	3 (4)
% Infestation (Flushes to sample)	55	45	344	279		10			

G= Girón, Santander. D= Dibulla, Guajira.
Bouvet & Hochmaier (2020) recommend values per Ha, except for yellow traps, with 5 in plots of less than 5-ha.
Stansly et al. (2010) data for 2-ha, explicitly the data is not presented by the author for 1-ha but based on conversion of an average lot of 50 acres = 20 ha.

Table 3. Correlation matrix of sampling methods for adults and immatures of the Asian citrus psyllid *Diaphorina citri*.

Girón, Santander						
Correlation \ p n=461	Yellow traps	Stem tap	Nymphs on flushes	Eggs on Flushes	Sweep net	Individuals mean
Yellow traps		**0,0001**	**5,6 e-08**	**8,8 e-09**	**1,78e-08**	7,5
Stem tap	**0,178**		**0,017**	**0,024**	0,083	0,07
Nymphs per flushes	**0,249**	**0,110**		**1,50e-28**	0,236	9,64
Eggs per flushes	**0,264**	**0,104**	**0,485**		0,069	6,41
Sweep net	**0,311**	0,098	0,067	0,102	0	0,286
Dibulla, La Guajira						
Correlatión \ p n=688	Yellow traps	Stem tap	Nymphs on flushes	Eggs on flushes		Individuals mean
Yellow traps		0,80	**4,5 e-06**	**0,0023**		0,42
Stem tap	0,009		0,97	0,916		0,006
Nymphs per flushes	**0,173**	-0,001		**7,39e-16**		1,24
Eggs per flushes	**0,115**	-0,004	**0,30**			1,65

It is recommended that for weekly monitoring, one of the three methods of adult and immature flush shoot inspection be used. In the case of tapping + shoot inspection, the costs would be 715,000 per year (13,750 per week). for sweep net + shoot inspection would be 800,000 (15,384 weekly), and for yellow sticky traps + shoot inspection, 1,016,000 per year (19,538 weekly). Although these costs will decrease significantly if sampling methodologies for *D. citri* are included in orchards where phytosanitary monitoring is already carried out on a weekly basis, because some materials are also used to monitor other arthropod pests and the time spent moving within the orchard would be the same, with optimization of personnel, time, and materials. It is important to emphasize that the samples in any of the methods must be well distributed and traps must be placed both inside and at the edge of the orchard due to the differences detected between plots of 0.2 ha.

In conclusion, the yellow sticky trap sampling method shows the highest sensitivity, and in times of low or no presence of shoots, is the best option for estimating the adult population of *D. citri* using 3 to 5 traps per 2 ha plot. This method can be easily adopted by the farmer or extensionists with prior training in the recognition and identification of *D. citri* adults and its costs are comparable to the other monitoring methods. The orientation of the traps is relevant in some cases and should consider possible sources of psyllid infestation as well as the prevailing wind direction.

The percentage of infestation in shoots is an easy sampling method to evaluate the population of immature *D. citri*. For a representative sampling, it is recommended to evaluate 45 to 55 shoots well distributed in the 2-ha plot.

For weekly monitoring, it is recommended to implement one of the three adult sampling methods, preferably yellow sticky traps and inspecting immature shoots, in addition to phytosanitary sampling for other citrus arthropod pests.

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