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Employing a Non-destructive Method for the Estimation of Foliar Area of Quina (*Cinchona officinalis*) †

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Abstract: Leaf area is related to tree growth, water balance and mechanical resistance to physical and biotic agents. Given its importance, the purpose of the study was to compare two nondestructive methods of leaf area estimation using the free software ImageJ vs graph paper, in seedlings of quina tree. Three young and mature leaves were evaluated on 18 quina seedlings. Descriptive statistics were obtained and both methods were compared using the Kruskal Wallis test and a regression equation was estimated based on leaf width and length.

Keywords: graph paper, leaf area, cinchona, allometric model.

1. Introduction

Maximum adult tree height, seed volume, wood density and specific leaf area are essential factors in explaining tree growth and mortality rates. Likewise, wood density and leaf area are related to water balance, mechanical resistance to physical and biotic agents, and tree architecture [15].

Leaf area is the surface where energy and matter are exchanged between the plant and the atmosphere, it is an essential factor in vegetation structure in agriculture, ecology, and forestry, to model tree growth. There are several challenges in leaf area estimation methods, to seek efficiency and feasibility [4], as they must be simple, fast and accurate [8]. [7] pointed out that leaf area measurements are used to evaluate plant growth, photosynthetic rates, and plant transpiration, constituting an important indicator of productivity.

There are several methods to determine leaf area, which are classified as destructive and non-destructive, as well as direct and indirect methods [18]. The most common leaf area measurement methods are gravimetry, manual planimetry, photoelectric and graph paper [12]. Measurements are generally performed by direct methods, which involve the destruction of the material [2] through methods and equipment that are time consuming and not feasible for practice, as well as there may be inexpensive and fast methods such as the use of free image processing [17].

The methods in which digital imaging is used can be destructive or non-destructive, and some software packages can be downloaded for free, which can contribute to the development of research projects [11]. There are several studies where the use of software and other methods have been compared, obtaining favorable results for the use of programs [10,20,11].

Leaf area estimation has also been developed from models based on linear measurements such as leaf length and width [9], there are several studies for agronomic species but there are very few studies for forest species [1]. Linear measurements performed on

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leaf blades are non-destructive, easy to perform and repeatable over time, and the high correlation between leaf dimensions makes them appropriate and reliable for estimation procedures [3].

Cinchona officinalis is a species known as "quina tree", being very characteristic for being represented in the national coat of arms of Peru, as a symbol of the plant kingdom. It has cultural, medicinal, environmental-forestry and economic importance within the country. This species possesses metabolites and alkaloids that have contributed in the treatment of malaria, provides ecosystem services as a hydrobiological and climate regulator, and has a good wood quality for boards and housing construction [5]. Due to its wide range of benefits, its population has been threatened, which caused reduction in its population and low natural regeneration [19]. It is currently listed as a vulnerable species in the IUCN (International Union for Conservation of Nature) red book [13].

Therefore, the objective of this study was to determine a fast and reliable method to characterize quina seedlings leaf area. We employed digital images processed with ImageJ software and the standard millimeter paper method.

2. Materials and methods

2.1. Location of the study

The study was conducted at the La Molina Agricultural Experiment Station of the National Institute for Agrarian Innovation (INIA in Spanish) in Lima, Peru. Measurements were made on 108 plant leaves of 18 seedlings of quina from the department of Cajamarca.

2.2. Procedure

Leaf area estimation of three young and three mature leaves of each seedling was carried out using two non-destructive methods, the ImageJ software (https://imagej.net/) and a A4 millimeter paper.

2.2.1. *Leaf area estimation with ImageJ Software*

The leaves of the seedlings were photographed at an average perpendicular distance of 30 cm with a Canon Rebel EOS T5 digital camera of 18 megapixel resolution, placing the leaf blades on top of an A4 bond sheet. After the photographic work, the images were subjected to width, length and leaf area measurements using ImageJ software. Following the methodology used by [11] in which (1) the leaf was photographed with the camera in a horizontal position, the leaf completely flat on the work surface and a reference scale, (2) The image was opened in ImageJ: File > Open, (3) Remove superfluous targets: Image > Crop, (4) Set scale: Analyze > Adjust Scale, (5) Adjust Contrast: Image > Type > 8-bit Image > Adjust > Threshold, (6) Calculate Area: Analyze > Analyze Particles.

2.2.2. *Estimation of leaf area in A4 millimeter sheets*

Leaf area estimation measurements were taken through millimetric sheets, delimiting the contour of the plant leaves on A4 millimetric sheets positioned at the back. The data of width, length and leaf area taken were stored in an Excel spreadsheet, where they were later analyzed by descriptive statistics and statistical tests.

2.2.3. *Estimation of leaf area in A4 millimeter sheets*

The Kruskal Wallis test, a nonparametric statistic where the following hypotheses will be put forward, will be implemented:

H0*: There is no difference in area measurements between the two methods*
H1: *There are differences in area measurements between the two methods.*

Considering a significance level of = 5%, the test statistic:

$$H = \frac{1}{S^2}\left[\sum \frac{{R_i}^2}{n_i} - \frac{n(n+1)^2}{4}\right] \sim X^2_{(1-\alpha,k-1)}$$

Where:

n: *Total number of the sample*

R_i: *Sum of the ranks of each sample*

n_i: *Number of observations for each sample*

k: *Number of treatments or groups*

S^2: *Total variance of the sample*

2.3. Data analysis

Descriptive statistics were developed using the Minitab 19 program, where the total number of leaves evaluated (N), the arithmetic mean, the standard deviation (SD), the minimum and maximum values found in young and mature leaves were determined.

The data collected did not meet the criteria of normality and homoscedasticity, so it was decided to perform a nonparametric test, where it was corroborated that there is no significant difference in the use of both methods. Finally, the design of linear and quadratic models was used for the estimation of leaf area, developed from measurements of leaf length and width.

3. Results and discussions

The total number of leaves evaluated for quina in the present study was 108 (54 young leaves and 54 mature leaves). Measurements of length, width and leaf area estimation were taken using two methodologies: ImageJ and graph paper.

Table 1 shows leaf dimensions measured by leaf type and method used according to the ImageJ software and the millimeter leaves. We here found the variables of total number (N), mean, standard deviation, minimum and maximum. We found that the range of the mean of the leaf area estimation is greater in mature leaves than in young leaves, this may be because it was observed that mature leaves had pests that caused a decrease in leaf area and thus a greater variation in the measurement.

Likewise, it was observed that the mean in relation to its length is out of the established by [14], who mentions that the length of the single leaf varies from 6-11cm and the width from 3-5cm. This may be due to the seedlings being in different environmental conditions and exposed to different substrate compared to their original habitat.

In relation to the coefficient of variation, in the case of leaf area, it ranged from 65.44 (ImageJ) to 60.67 (graph paper) in young leaves, and 38.67 to 38.75 in mature leaves. The highest value was for young leaves; this may be due to the variability of the size of quina leaves in each method. On the other hand, these values were within those found by [11], where he studied the comparison between the LI-COR 3100 and Image J methods in oat leaves.

Table 1. Summary statistics of young and mature leaf dimensions measured by ImageJ software and millimeter leaves.

Variable	Method	Young leaves				Mature leaves			
		Average	Stand. Dev.	Min.	Max.	Media	Stand. Dev.	Min.	Max.
Área	Image J	80.72	52.82	7.62	257.21	255.28	98.73	100.3	590.55

	Graph paper	80.75	48.99	13	251.75	245.86	95.28	89.75	555
Length	Image J	11.72	3.72	4.23	20.91	21.66	3.73	14.76	30.3
	Graph paper	12.24	3.42	5	21	21.43	3.93	11.9	29
Width	Image J	8.59	3.03	2.64	17.58	16.17	3.52	8.89	25.98
	Graph paper	9.17	3.09	3.5	19	15.76	3.32	8	23

The statistical comparison was performed using a nonparametric test since the data collected are not normally distributed and do not meet the assumption of homoscedasticity through constant variance.

H_0: *There is no difference in area measurements between the two methods.*
H_1: *There are differences in area measurements between the two methods.*

Under the above hypothesis, using the Kruskal Wallis test, a p-value = 0.986 was obtained, so we can conclude that sufficient statistical evidence was found to not reject H_o, i.e., there are no significant differences in the area measurements between the ImageJ method and the millimeter method. [16] performed the comparison between Licor 3000 integrator and Macf-IJ and found the regression analysis (R^2) between both methods was 0.999. Also, they indicated that Macf-IJ is a fast and accurate method to measure leaf area, dimensions and color using scanned images and digital photographs.

3.1. Leaf area estimation model:

Table 3. Expression of the regression model in the estimation of leaf area.

Expression of the model	Coefficients of determination	
	R^2	$Radj^2$
AF = -122.469 + 7.371L + 13.37W	0.9486	0.9476

3.2. Interpretability of the model:

Length: *For every centimeter of leaf length, the final area increases by 7.371 cm2.*
Width: *For each centimeter of leaf width, the final area increases by 13.37 cm2.*

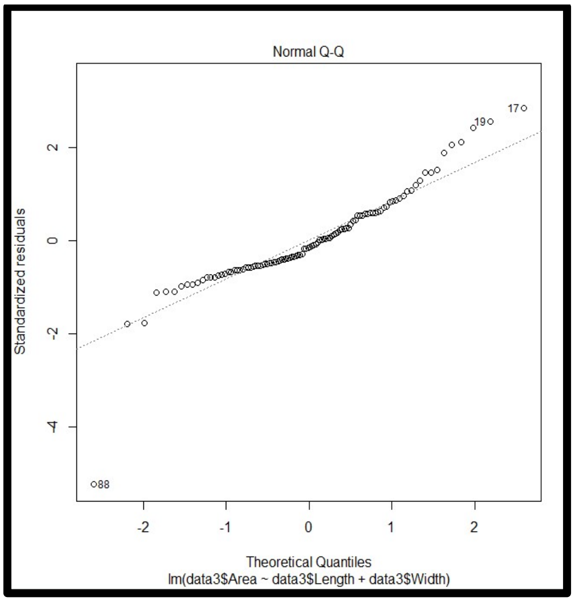

Figure 1. Fitted line graph of the leaf area of quina.

Table 4. Results obtained from the linear model implemented in R

	Estimator	SD	T value	Significance
Intercept	-122.469	7.260	-16.869	***
Length	7.371	1.380	5.341	***
Width	13.370	1.734	7.710	***

R Squared = 0.9486 Adjusted R Squared = 0.9476

A linear model was implemented with the variables length and width, finding favorable results when estimating the leaf area. This model explains 94.86% of the total information of the variable in question as the independent variables are strongly related to the target variable. These adjustments with linear and quadratic models were also studied in the potato variety "agata" where an equation adjusted with the length of the leaves evaluated was determined [6].

ImageJ has been compared with other paid software, where it has been concluded that it is possible to find leaf area and leaf lamina dimensions using the free ImageJ software, resulting practical and efficient. According to [20], ImageJ is fast and simple to use, and do not lose precision compared to Image-Pro PLUS and AFSoft.

[11] compared the methods of ImageJ and the LI-COR 3100 leaf area meter on white and black oats and concluded that there is no significant difference between the two methods. They also indicated that ImageJ can be used instead of the meter. Similarly, [10] compared the ImageJ software method and the LI3100 LI-COR leaf area integrator in two bean genotypes (black CHP 99-54 and carioca SCS 202 Guará), proving that the ImageJ program can be used as a substitute for the leaf area integrator, being the digital image method an easy, fast, economical and non-destructive way to perform leaf area evaluations.

4. Conclusions

There is no significant difference between leaf area estimation using ImageJ software and graph paper. ImageJ is an excellent option for leaf area estimation, being a non-destructive, free, practical, and efficient method, allowing significant savings in time and cost. The allometric models that best fit in the case of leaf area estimation for quina is the quadratic one.

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