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Chlorophyll measurements in *Alstroemeria* sp. using *SPAD-502* meter and the color space CIE L*a*b*, and its validation in foliar senescence

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Abstract

Our research aimed to study the correlation between the *SPAD-502* readings and the color space CIE L*a*b* values in two cultivars of *Alstroemeria* sp. during leaf senescence and to evaluate the statistical criteria used in the selection of the best fit calibration functions. We demonstrate the importance of the Akaike information criterion and the parsimonious function besides the coefficient of determination. The reliability of the functions was tested by *Student*'s *t*-test comparison between the chlorophyll (Chl) estimated from SPAD readings and their chemical concentrations. Polynomial and Hoerl function described well the changes in Chl *a* and total Chl (*a*+*b*) during senescence, but calibration functions are required to perform for each cultivar. We demonstrated that CIE L*a*b* system is reliable to estimate SPAD reading at stages of leaf senescence of *Alstroemeria* sp. and can be used instead of *SPAD-502*.

Keywords: Akaike; color measurements; greenness; ornamentals; photosynthetic pigments; SPAD calibration.

Introduction

Chemical measurement of Chl concentration in the leaf is an important parameter used to evaluate photosynthetic capacity, nitrogen and protein content, and overall plant health status (Ling *et al.* 2011). Photosynthetic pigments, such as Chl *a*, Chl *b*, xanthophylls and carotenoids (*x*+*c*), are inserted into thylakoid membranes, particularly in PSI and PSII, which are indispensable for photosynthesis. Chl is a molecule formed by a tetrapyrrole ring in which the radical of position 3 of the tetrapyrolic group can be -CH_3 (Chl *a*) or -CHO (Chl *b*) and a phytol chain (Kuai *et al.* 2018). Chls typically have two types of absorption in the visible spectrum, one in the blue light region (400–500 nm), and another in the red area of the spectrum (600–700 nm); however, they reflect the middle

Highlights

- Use of Akaike criterion for a proper selection of a calibration function
- The CIE L*a*b* system can be used to monitor leaf senescence
- The *SPAD-502* meter is accurate to estimate chlorophyll in *Alstroemeria* sp. leaves

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Abbreviations: A – absorbance; AIC – Akaike information criterion; Chl – chlorophyll; CI – color index; CIE – Commission internationale de l'éclairage; LED – light-emitting diode; R^2 – coefficient of determination; SPAD – Soil plant analysis development.

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part of the spectrum that corresponds to the green color (500–600 nm) (Chen 2014). Leaf greenness is an indirect indicator of the relative concentration of photosynthetic pigments of the leaf. Chemical quantification of Chls is commonly done photometrically in organic solvent extracts, such as acetone, ethanol, dimethylformamide (Lichtenthaler 1987, Ling *et al*. 2011), and although this method is accurate, it is destructive, time-consuming, and uses toxic chemicals.

For physiological studies, it is ideal to monitor changes in Chl content in the same leaf tissue to evaluate the effects of treatments, nutritional status, senescence, or abiotic stress over time. In postharvest studies, the measurement of Chl of ornamental foliage is a subject of interest (Lin *et al.* 2010, Li *et al.* 2017). The use of nondestructive methods is very appealing because they are inexpensive, less time-consuming, and the same tissue can be monitored more precisely (Yuan *et al*. 2016a,b). At a concentration of 80% acetone–water, the wavelengths of the absorption maxima of Chl *a* are 430 and 663 nm; and for Chl *b*, it is 460 and 643 nm (Lichtenthaler 1984).

Following the maker manual, the *SPAD-502* meter, *i.e*., 'Soil plant analysis development' (*Konica-Minolta*, Japan) is a nondestructive and portable method that provides a relative greenness of the leaf, based on two light-emitting diodes (LED) and a silicon photodiode receptor. This technique assumes that acetone Chl extract has absorbance peaks in the blue (400–500 nm) and red (600–700 nm) regions with no absorbance in the near-infrared region. LEDs in the illuminating system of the *SPAD-502* Chl meter emit red (peak wavelength: approx. 650 nm) and infrared (peak wavelength: approx. 940 nm). The light which passes through the leaf strikes the receptor which converts the transmitted light to analog electrical signals, and they are used to calculate a SPAD value. The relationship between leaf Chl concentrations and *SPAD-502* values has been demonstrated (Minolta 1989, Süß *et al*. 2015). This device has been used to measure the leaf Chl concentration in *Prunus virginiana* L., *Viburnum lentago* L., *Oryza sativa* L., and *Arabidopsis thaliana* (Ling *et al.* 2011, Donnelly *et al.* 2020, Liu *et al.* 2020). For each species, it is necessary to derive a calibration curve with the SPAD value and its corresponding Chl content (Li *et al*. 2017, Primka and Smith 2019).

In horticultural postharvest research, the color change is an important parameter commonly evaluated using the color space CIE (Commission internationale de l'éclairage), the authority that defines color in terms of CIE L*a*b*. The space of color L*a*b* consider the 'opponent color theory' where two colors (red and green or yellow and blue) cannot be at the same time. Therefore, L* = luminosity, a* = coordinates red/green (+a indicates red, –a indicates green) and b* = coordinates yellow/blue (+b indicates yellow, –b indicates blue) (Gevers and Smeulders 1999, Gómez-Polo *et al*. 2016).

Leaf senescence is the last stage of leaf development; it is a genetically controlled process and yellowing is the main symptom of senescence due to the degradation of Chls and chloroplasts (Gan 2018). Senescence is characterized by the translocation of NO_3^-, NH_4^+, amino acids, carbon, and minerals from the senescent leaf to the growing parts of the plant (Dangl *et al.* 2000, Woo *et al*. 2013, Zhao *et al.* 2018). Therefore, the reduction in foliar Chls is an important parameter to study and monitor senescence (Matile *et al.* 1996).

The color spaces (L*, a*, b*), measured by CIE L*a*b* have been used to evaluate the color of some horticultural products (flowers, fruits, vegetables) (Chiabrando and Giacalone 2018, Teppabut *et al.* 2018, Gracia-Romero *et al.* 2019). Some studies indicated that the color space CIE L*a*b* can be used to study senescence (Teppabut *et al.* 2018, Luo *et al.* 2020) only when the factors that influence the color perception (light intensity, saturation, and angle) are controlled and homogenized (McGuire 1992, Gevers and Smeulders 1999, Gómez-Polo *et al.* 2016).

Although SPAD and CIE L*a*b* color are two nondestructive techniques to measure the greenness of a leaf, the functions that related them to the chemical content of Chls have not been statistically tested and validated for leaf senescence studies in specific ornamental species.

Alstroemeria sp. is an important ornamental vase flower, which is widely commercialized in Europe and America and occupies the sixth place among the most popular cut flowers around the world (Lim *et al.* 2012, Samaniego-Gámez *et al.* 2012). *Alstroemeria* sp. have an average vase life spam of 10 to 21 d, but it faces the problem that leaves of the inflorescence become yellow and senescent approximately before (3–4 d) flower wilting, and these physiological conditions carry a negative impact at postharvest (Chanasut *et al.* 2003, Galati *et al.* 2017, Langroudi *et al.* 2019). In our group, we are studying some treatments to delay leaf senescence and we have faced the necessity to evaluate fast and accurately the greenness and Chl content of the leaves in a nondestructive manner. Therefore, we propose the present research to analyze calibration functions for *SPAD-502* meters based on three statistical selection criteria and study the relationship between the color space CIE L*a*b* and the SPAD values. To validate the utility of the calibration equations presented, for the first time, color space CIE L*a*b* is used to derive SPAD values and Chl amount [Chl (*a*+*b*)] in the context of the foliar senescence in two contrasting cultivars of *Alstroemeria* sp. 'Tonatiuh' and 'Fogo'.

Materials and methods

Plant material: We used two contrasting cultivars of *Alstroemeria*, a red 'Fogo' and a yellow 'Tonatiuh' (Fig. 1*A*). Flowers were obtained from the grower company *COXFLOR* at Villa Guerrero, Estado de México (latitude 18.96, longitude: –99.64, 18°57'36"N, 99°38'24"W). One hundred floral stems of 80-cm length were harvested in the morning (7:00–8:00 h), at the close bud flowers stage (Fig. 1*B*). Flower stems were placed in plastic sealed black bags and dry transported (3 h, 22–23°C) to the laboratory at the Colegio de Postgraduados, Montecillo Campus, Texcoco, State of Mexico (19°27'50.78"N, 98°54'14.91"W).

Experiment for vase foliar senescence: *Alstroemeria* sp. develops a compound umbel type inflorescence. An umbel is an indeterminate, open flat-topped inflorescence whose pedicels and peduncles (rays) arise from a 'common point'; when it is compound, each primary ray terminates by the second umbel (Fig. 2*A*). A total of 80 flower stems of each cultivar were homogenized, to 50 cm in length, measured from the 'common point' to the base of the stem. The leaves, below the fourth node, counting from the 'common point' downwards, were removed to avoid the contact of the basal leaves with the base solution. Two flowering stems were placed in each of 500-mL capacity vases, filled with 400 mL of tap water, and sealed with parafilm to prevent evaporation. The third leaf of each stem was the experimental unit. A total of 35 vases with two flowers stems (70 stems per cultivar) were distributed completely randomly in a room with indirect natural light under circadian illumination 12 h dark/12-h light [PAR of 50 µmol(photon) m^{-2} s^{-1}], day/night temperature of 22/19°C; relative humidity of 44% for 23 d. All samples and measurements were taken from the third leaf below the umbel.

Fig. 1. *Alstroemeria* sp.: (*A*) leaf and red flowers of 'Fogo', (*B*) leaf and yellow flowers of 'Tonatiuh', (*C*) 'Fogo' bud flowers at harvest time, (*D*) 'Tonatiuh' bud flowers at harvest time.

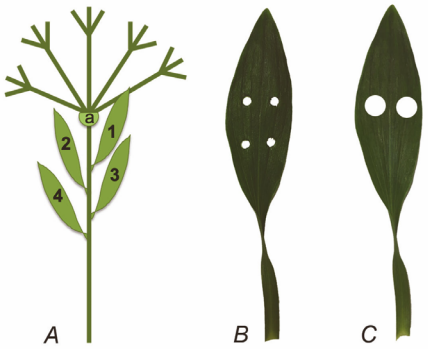


Fig. 2. (*A*) Morphology of the inflorescence of *Alstroemeria* sp. The flowering stem of *Alstroemeria* sp. is botanically classified as a compound umbel, where a simple umbel is identified as an indeterminate, open flat-topped inflorescence, whose pedicels and peduncles arise from a 'common point' (a) and the compound umbel is when each peduncle terminates in a secondary umbel. For the experiment, leaves were counted down from the 'common point' to the base of the stem and leaves below the 4th node were removed. (*B*) Regions (white disks) where the *SPAD-502* meter measurements were performed. (*C*) Regions (white disks) where the color space CIEL*a*b* measurements were performed.

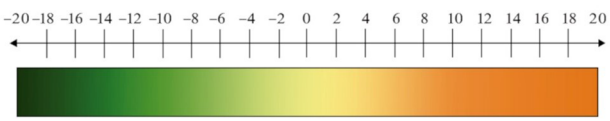
***SPAD-502* meter measurements**: Leaf Chl readings were performed with the *SPAD-502* meter (*Konica-Minolta*, Japan) in four areas (6 mm^2) of the central part of the third leaf below the umbel, per floral stem, as illustrated in Fig. 2*B*. The four measurements were averaged to obtain one mean value per stem. Measurements were made daily for 23 d to obtain a range of pigmentation levels, from green to pale yellow.

Chemical quantification of Chls was performed on the same four areas where the *SPAD-502* readings were carried out. Leaf tissue was harvested with a core borer that yielded 0.5-cm diameter discs; Chl of four discs was extracted in 3 mL of 80% acetone–water. After centrifugation at 1,844 × *g* (*Eppendorf 5804R*) for 5 min, the absorbance (A) of the supernatant was read at 470, 647, and 663 nm using a spectrophotometer (*Genesys 10*, *Thermo Scientific*, USA), and Chls were calculated [µg mL^{-1}] according to Lichtenthaler (1987): Chl *a* = $12.25A_{663} - 2.79A_{647}$, Chl *b* = $21.5A_{647} - 5.10A_{663}$, Chl (*a*+*b*) = $7.15A_{663} + 18.71A_{647}$. The results were converted to µg cm^{-2}, considering the dilution volume of the extract (3 mL) and the area of four discs (0.7854 cm^2), using the formula $Area^2 = \pi r^2 \times 4$.

Color measurements were performed under the CIE L*a*b* system, with an *NR20XE* Precision Colorimeter (*Shenzhen ThreeNH Technology*, China) on the same leaf regions where the *SPAD-502* measurements were made, daily for 23 d. Since the area (133 mm^2) covered by the colorimeter was larger than that covered by the *SPAD-502*, only two-color readings were done and averaged per leaf as shown in Fig. 2*C*.

The values obtained from the CIE L*a*b* color system were used for the determination of the color index (CI) with the formula provided by Vignoni *et al.* (2006): CI = (a × 1,000)/(L × b). According to Vignoni *et al.* (2006), the CI can be used like a greening indicator as specified in the following chart.

CI	Color	
–20 to –2		deep green to yellowish-green
–2 to 2		greenish-yellow
2 to 20		pale yellow to intense orange



Relationship between *SPAD-502* meter and Chl content: Leaves, from green (56 SPAD values) to pale yellow (2 SPAD values) were used. SPAD readings and chemical measurements of Chls concentration [μg cm^{-2}] were performed for each leaf. By plotting these two datasets against each other, we studied the possible relationship between them for each cultivar with the *Curve ExpertPro Professional version 2.6.5* program. To select the calibration function that better described the relationship between SPAD readings and the Chl concentrations, the quality was evaluated according to Bozdogan (1987) considering the following parameters: (*1*) parsimonious, *i.e*., with few parameters, (*2*) coefficient of determination (R^2) closer to one, and (*3*) the lowest Akaike information criterion (AIC) (Bozdogan 1987). The same parameters were used to evaluate the relation between the values obtained with the colorimeter *NR20XE* and the *SPAD-502* meter for each cultivar.

Definition of the stages of leaf senescence: Leaves of *Alstroemeria* sp. showed a gradual yellowing during natural senescence, which covered a whole range of yellowing from green (56 SPAD value) to advanced senescence (2 SPAD value). Stages of leaf senescence are widely defined in terms of SPAD values (Lohman *et al.* 1994, Otegui *et al.* 2005). To the present research, four stages of senescence were identified for 'Tonatiuh' and 'Fogo': green (G) from 56 to 43 SPAD values, early senescence (S1) from 42 to 29 SPAD values, intermediate senescence (S2) from 28 to 15 SPAD values, and advanced senescence (S3) from 14 to 2 SPAD values. In our study, advanced stages of senescence (S3) did not include values < 2 because the *SPAD-502* meter used shows an error interval of ± 1.0 units by the manufacturer manual (Minolta 1989).

Statistical validation of the *SPAD-502* meter calibration functions in stages of leaf senescence: The calibration functions were statistically validated in a model of leaf senescence in two cultivars of *Alstroemeria* sp. 'Fogo' and 'Tonatiuh'. A comparison of the data converted to SPAD values, using the calibration functions with those derived chemically from extracted Chls, was performed by a *Student*'s *t*-test ($p \leq 0.05$, $n = 10$ per senescence stage) to confirm the similarity between them. The null hypothesis was: 'the chemical quantification of photosynthetic pigments from the leaves of the two *Alstroemeria* sp. cultivars is similar to the predicted values obtained with the selected function using the *SPAD-502* meter'. To test the null hypothesis, the homogeneity of variances was corroborated according to the *Bartlett* test and the normal distribution of the data was verified according to *Shapiro*'s test using the program *R version 3.5.1* (2018).

Results

Relationship between *SPAD-502* meter and Chl content: The average SPAD values per leaf were graphed against its corresponding chemical concentrations [μg cm^{-2}] of Chl *a*, Chl *b*, and total Chl. Statistical parameters of the relationship between SPAD readings and the spectroscopic measurements of photosynthetic pigments in cultivars of *Alstroemeria* sp. leaves 'Tonatiuh' and 'Fogo' are shown in Table 1. The R^2 obtained for Chl *a*, Chl *b*, and total Chl in both cultivars were > 0.92; for 'Tonatiuh' the R^2 was 0.927–0.962, in contrast, the functions for 'Fogo' showed higher R^2 (0.970–0.987), and for both cultivars, the functions Horel, Polynomial, and Power showed the highest values. Considering the AIC and R^2, the best fit in 'Tonatiuh' was shown by Hoerl ($y = ab^x x^c$) for all Chls, in contrast, for the 'Fogo', the Power function showed best fit for Chl *a* and total Chl and the Polynomial function ($y = a + bx + cx^2$) for Chl *b* (Table 1).

Validation of the calibration functions of *SPAD-502* meter values and Chl concentration: The validation of the calibration functions of the *SPAD-502* meter was performed comparing the estimated Chl content with the SPAD values and the chemical determination of Chls at four stages of development (G, S1, S2, and S3). Before performing a mean comparison test, the data need to meet the criteria of the null hypothesis, variance homogeneity, and normal distribution. For 'Tonatiuh', the *Student*'s *t*-test showed similarity between the data converted with the calibration functions Horel and Polynomial with the chemical measurements of Chl *a* and total Chl, indicating that these equations are reliable to convert SPAD values into Chl measurements (Table 2).

In contrast, the Power function also showed similarity between the data converted with it and the chemical measurements of Chls in 'Fogo' (Table 3). The Linear function was not consistent in all stages of senescence. For 'Tonatiuh', Chl *a* of S1 was statistically different, but for 'Fogo', the pigment was different in G, S1, and S2. The variance homogeneity was not demonstrated for both cultivars in G and S3.

No statistical function was found to estimate properly the Chl *b* in all stages of senescence. In particular, Horel was the better model to estimate Chl *b* only in S1 in 'Tonatiuh', and for S2 and S3, all the models proposed are good to estimate the pigment in both cultivars (Tables 2, 3).

For both cultivars, the percentage of the difference between the calculated and chemical means (Δ) of all pigments showed large variations, 'Tonatiuh': –9.73% to 22.85% and 'Fogo': –5.09% to 32.81%, with a tendency to increase at advanced senescence (S3) (Tables 2, 3) but they were not coherent with the mean comparison test (*Student*'s *t*-test).

Color measurement and color index with CIE L*a*b*: Relationships between SPAD values and color indexes (CI) for 'Tonatiuh' and 'Fogo' are shown in Table 4. Three mathematical functions for each cultivar were selected accordingly with the criteria of parsimony, R^2 close to one, and the lowest AIC (Table 4). For 'Tonatiuh', the best fit function was Polynomial, and for 'Fogo' was Horel. With these functions, it is possible to convert CI readings into SPAD values or *vice versa* and then estimate the Chl content from a CI value. The utility of the selected functions is illustrated in Tables 5, 6 for both cultivars. Four stages

Table 1. Statistical parameters of the relationship between SPAD readings and the spectroscopic measurements of photosynthetic pigments of *Alstroemeria* sp. 'Fogo' and 'Tonatiuh' leaves. Four models (Hoerl, Polynomial, Power, Linear) are presented with its corresponding function, Akaike information criterion (AIC), and determination coefficient (R^2), n = 70.

Cultivar	Chls	Model	Function	AIC	R^2
'Tonatiuh'	Chl *a*	Hoerl	$y = 0.433 \times (1.015^x) \times (x^{0.874})$	119.695	0.962
		Polynomial	$y = 0.627 + 0.227x + 0.007x^2$	119.391	0.960
		Power	$y = 0.102x^{1.441}$	123.331	0.959
		Linear	$y = -3.040 + 0.618x$	150.796	0.936
	Chl *b*	Hoerl	$y = 0.288 \times (1.018^x) \times (x^{0.732})$	16.203	0.953
		Polynomial	$y = 0.471 + 0.097x + 0.003x^2$	17.670	0.952
		Power	$y = 0.052x^{1.406}$	21.332	0.948
		Linear	$y = -1.156 + 0.270x$	44.697	0.927
	Chl (*a*+*b*)	Hoerl	$y = 0.714 \times (1.016^x) \times (x^{0.827})$	172.212	0.959
		Polynomial	$y = 1.098 + 0.324x + 0.010x^2$	173.396	0.959
		Power	$y = 0.152x^{1.433}$	176.143	0.956
		Linear	$y = -4.196 + 0.888x$	203.813	0.934
'Fogo'	Chl *a*	Hoerl	$y = 0.124 \times (1.000^x) \times (x^{1.392})$	23.839	0.987
		Polynomial	$y = -0.162 + 0.305x + 0.006x^2$	25.501	0.987
		Power	$y = 0.123x^{1.397}$	21.716	0.987
		Linear	$y = -2.994 + 0.616x$	76.667	0.971
	Chl *b*	Hoerl	$y = 0.167 \times (1.011^x) \times (x^{0.977})$	–88.671	0.987
		Polynomial	$y = 0.265 + 0.126x + 0.003x^2$	–90.921	0.987
		Power	$y = 0.068x^{1.339}$	–84.688	0.986
		Linear	$y = -1.024 + 0.267x$	–34.504	0.970
	Chl (*a*+*b*)	Hoerl	$y = 0.262 \times (1.004^x) \times (x^{1.251})$	70.818	0.987
		Polynomial	$y = 0.103 + 0.431x + 0.008x^2$	70.675	0.987
		Power	$y = 0.190x^{1.379}$	69.347	0.987
		Linear	$y = -4.018 + 0.883x$	125.076	0.971

of senescence (G, S1, S2, S3) were well characterized, using SPAD values to estimate CI and Chl contents.

Discussion

Foliar senescence has been studied in natural biological models, induced by darkness, in mutants, or deciduous trees (Wojciechowska *et al.* 2018, Chen *et al.* 2021, Kang *et al.* 2021). One of the first events of foliar senescence is the degradation of Chl, reflected as the yellowing of the leaf; this change of color is associated with the loss of greenness.

In this work, we identified three stages of natural foliar senescence (S1, S2, S3) in *Alstroemeria* sp. based on SPAD values. Foliar senescence studies require the use of nondestructive methods to evaluate changes in photosynthetic pigments. The *SPAD-502* meter was used originally to assess the yellowing of rice leaves under different conditions as an indicator of N deficiency (Markwell *et al.* 1995, Jhanji and Sekhon 2018). Due to the approximation offered by the SPAD readings with the content of Chls, this portable meter could be an accurate tool depending on the calibration function used. The calibration of the instrument is performed by plotting a dataset of SPAD readings against spectrophotometric measurements using pigment extracts ([μg mg^{-2}] or [μg cm^{-2}]), to assess possible relationships between them (Takebe and Yoneyama 1989, Lin *et al.* 2010, Primka and Smith 2019). In previous reports, the criteria to select the best calibration function were based on R^2 (*Acer* R^2 = 0.89, *Quercus* R^2 = 0.89, *Cronus* R^2 = 0.67, *Eperua* R^2 = 0.78, *Hymenaea* R^2 = *0.91*, *Glycine* R^2 = *0.96*, *Arabidopsis* R^2 = *0.99*; Markwell *et al.* 1995, Coste *et al.* 2010, Ling *et al.* 2011, Li *et al.* 2017). For *Alstroemeria* cultivars, the R^2 was > 0.92, obtained with Hoerl, Polynomial, Power, and Linear functions. However, the statistical analysis of our research demonstrated that a high R^2 is not the sufficient criterion to select a reliable calibration function.

The correlation coefficient describes how strong is a linear relationship between two variables (Ozer 1985). In contrast, the R^2 is the square of the correlation coefficient generally used to find the best fit often from a regression model, providing the percentage of variation in y explained by x-variables and it ranges from 0 to 1 (0 to 100% of the variation) (Nagelkerke 1991). However, we consider that the R^2 should not be the only criteria used to select a relationship function. The quality of statistical functions

Table 2. Statistical validation of the SPAD calibration functions in stages of leaf senescence of *Alstroemeria* 'Tonatiuh'. The amount of Chl *a*, Chl *b*, and total Chl estimated with the mathematical functions (Horel, Polynomial, Power, and Linear) was compared with its amount chemically measured in leaf tissue: green (G), early senescence (S1), medium senescence (S2), late senescence (S3). Data are means ± SE, *different letters* indicate significant differences between each function and the chemical measurement of Chl *a*, Chl *b*, and total Chl (*Student*'s *t*-test, $p<0.05$, $n = 10$). † – Shapiro, ** – Bartlett.

Chls	Model	G		S1		S2		S3	
		[μg cm^{-2}]	Δ [%]	[μg cm^{-2}]	Δ [%]	[μg cm^{-2}]	Δ [%]	[μg cm^{-2}]	Δ [%]
Chl *a*	Chemically	30.27 ± 0.67^{A}		17.60 ± 0.46^{A}		8.83 ± 0.55^{A}		2.99 ± 0.35^{A}	
	Hoerl	30.53 ± 0.36^{A}	0.86	16.88 ± 0.40^{A}	–4.09	8.63 ± 0.49^{A}	–2.27	3.71 ± 0.45^{A}	24.08
	Polynomial	30.56 ± 0.34^{A}	0.96	17.06 ± 0.41^{A}	–3.07	8.44 ± 0.51^{A}	–4.42	3.55 ± 0.41^{A}	18.73
	Power	30.47 ± 0.31^{A**}	0.66	17.48 ± 0.42^{A}	–0.68	8.31 ± 0.57^{A}	–5.89	2.83 ± 0.45^{A}	–5.35
	Linear	29.27 ± 0.23^{A**}	–3.30	18.92 ± 0.36^{B}	7.50	10.01 ± 0.64^{A}	13.40	2.98 ± 0.73^{A**}	–0.33
Chl *b*	Chemically	12.26 ± 0.27^{A}		7.09 ± 0.15^{A}		4.27 ± 0.24^{A}		1.52 ± 0.17^{A}	
	Hoerl	13.52 ± 0.16^{B}	10.28	7.51 ± 0.17^{A}	5.97	3.96 ± 0.21^{A}	–7.08	1.83 ± 0.20^{A}	20.19
	Polynomial	13.53 ± 0.15^{B}	10.42	7.61 ± 0.18^{B}	7.41	3.85 ± 0.22^{A}	–9.73	1.73 ± 0.18^{A}	13.76
	Power	13.50 ± 0.14^{B}	10.13	7.84 ± 0.18^{B}	10.65	3.80 ± 0.26^{A}	–11.01	1.32 ± 0.21^{A}	–13.03
	Linear	12.96 ± 0.10^{B†**}	5.77	8.44 ± 0.16^{B}	19.04	4.55 ± 0.28^{A}	6.62	1.48 ± 0.32^{A}	–2.90
Chl (*a*+*b*)	Chemically	42.53 ± 0.92^{A}		24.69 ± 0.61^{A}		13.09 ± 0.78^{A}		4.51 ± 0.53^{A}	
	Hoerl	44.10 ± 0.53^{A}	3.70	24.42 ± 0.57^{A}	–1.10	12.62 ± 0.71^{A}	–3.36	5.54 ± 0.65^{A}	22.85
	Polynomial	44.07 ± 0.49^{A}	3.62	24.66 ± 0.59^{A}	–0.13	12.28 ± 0.73^{A}	–6.17	5.28 ± 0.58^{A}	17.04
	Power	43.97 ± 0.45^{A**}	3.38	25.30 ± 0.60^{A}	2.44	12.08 ± 0.83^{A}	–7.74	4.13 ± 0.65^{A}	–8.44
	Linear	42.23 ± 0.33^{A†**}	–0.71	27.35 ± 0.52^{B}	10.78	14.56 ± 0.92^{A}	11.2	4.46 ± 1.04^{A**}	–1.21

Table 3. Statistical validation of the SPAD calibration functions in stages of leaf senescence of *Alstroemeria* 'Fogo'. The amount of Chl *a*, Chl *b*, and total Chl estimated with the mathematical functions (Horel, Polynomial, Power, and Linear) was compared with its amount chemically measured in leaf tissue: green (G), early senescence (S1), medium senescence (S2), late senescence (S3). Data are means ± SE, *different letters* indicate significant differences between each function and the chemical measurement of Chl *a*, Chl *b*, and total Chl (*Student*'s *t*-test, $p<0.05$, $n = 10$). † – Shapiro, ** – Bartlett.

	Model	G		S1		S2		S3	
		[μg cm^{-2}]	Δ [%]	[μg cm^{-2}]	Δ [%]	[μg cm^{-2}]	Δ [%]	[μg cm^{-2}]	Δ [%]
Chl *a*	Chemical	30.72 ± 0.65^{A}		16.02 ± 0.78^{A}		8.20 ± 0.51^{A}		2.56 ± 0.26^{A}	
	Hoerl	30.88 ± 0.37^{A}	0.52	17.03 ± 0.56^{A}	6.30	8.71 ± 0.54^{A}	6.22	3.04 ± 0.37^{A}	18.75
	Polynomial	31.13± 0.37^{A}	1.33	16.81 ± 0.56^{A}	4.93	8.79 ± 0.54^{A}	7.20	3.40 ± 0.37^{A}	32.81
	Power	30.87 ± 0.40^{A}	0.49	17.03 ± 0.55^{A}	6.30	8.71 ± 0.51^{A}	6.22	3.03 ± 0.37^{A}	18.36
	Linear	29.19 ± 0.28^{B**}	–4.98	18.01 ± 0.49^{B}	12.42	9.96 ± 0.58^{B}	21.46	3.02 ± 0.55^{A**}	17.97
Chl *b*	Chemically	12.84 ± 0.28^{A}		6.44 ± 0.31^{A}		4.27 ± 0.25^{A}		1.50 ± 0.14^{A}	
	Hoerl	13.85 ± 0.18^{B}	7.83	7.55 ± 0.24^{B}	17.09	4.11 ± 0.22^{A}	–3.77	1.73 ± 0.17^{A}	15.17
	Polynomial	13.83 ± 0.18^{B}	7.69	7.55 ± 0.24^{B}	17.11	4.07 ± 0.22^{A}	–4.77	1.76 ± 0.16^{A}	17.10
	Power	13.64 ± 0.16^{B}	6.21	7.71 ± 0.24^{B}	19.66	4.05 ± 0.24^{A}	–5.09	1.47 ± 0.18^{A}	–1.98
	Linear	12.94 ± 0.12^{A**}	0.80	8.09 ± 0.21^{B}	25.57	4.60 ± 0.25^{A}	7.74	1.59 ± 0.24^{A}	5.76
Chl (*a*+*b*)	Chemically	43.57 ± 0.92^{A}		22.46 ± 1.08^{A}		12.47 ± 0.75^{A}		4.06 ± 0.40^{A}	
	Hoerl	44.73 ± 0.56^{A}	2.68	24.57 ± 0.79^{A}	9.37	12.83 ± 0.75^{A}	2.91	4.77 ± 0.55^{A}	17.35
	Polynomial	44.96 ± 0.58^{A}	3.19	24.36 ± 0.79^{A}	8.43	12.85 ± 0.72^{A}	3.08	5.15 ± 0.53^{A}	26.81
	Power	44.51 ± 0.53^{A}	2.17	24.74 ± 0.80^{A}	10.15	12.76 ± 0.78^{A}	2.28	4.50 ± 0.55^{A}	10.71
	Linear	42.13 ± 0.40^{B**}	–3.31	26.10 ± 0.70^{B}	16.19	14.56 ± 0.83^{A}	16.76	4.61 ± 0.80^{A**}	13.43

can be evaluated according to Bozdogan (1987), who proposed the following parameters: (*1*) parsimonious, with few parameters, (*2*) an R^2 value closer to one, and (*3*) the lowest AIC. The AIC handles a compromise between the goodness of fit of the model and the complexity of the model, it is based on the entropy of the model's information and estimates the average value of the log-likelihood that allows selecting the model that approaches reality regardless of the parameters and the sample size (Akaike 1974). Coste *et al*. (2010) reported a calibration test for 13 species of tropical rainforest in Frech Guiana; considering the AIC, they concluded that for each

Table 4. Calibration functions that describe best the relationship between *SPAD-502* meter values and color indexes (CI) for *Alstroemeria* 'Tonatiuh' and 'Fogo' (n = 70). AIC – Akaike information criterion; R^2 – determination coefficient.

Cultivar	Model	Function	AIC	R^2
'Tonatiuh'	Polynomial	$y = 1.902 – 0.393x + 0.001x^2$	76.502	0.919
	Power	$y = –0.213x^{1.078}$	84.109	0.907
	Linear	$y = 1.162 – 0.314x$	77.541	0.915
'Fogo'	Hoerl	$y = –0.004 \times (0.962^x) \times (x^{2.612})$	90.146	0.947
	Power	$y = –0.115 \times (x^{1.256})$	93.862	0.885
	Linear	$y = 1.550 – 0.338x$	93.763	0.885

Table 5. Utility of the calibration functions for *SPAD-502* meter values and color index (CI) in four stages of senescence (G – green, S1 – early senescence, S2 – intermediate senescence, S3 – advanced senescence) of *Alstroemeria* 'Tonatiuh'. ω: the lower *SPAD-502* value was 2 considering the precision (± 1) of the instrument; ρ: $y = 1.902 – 0.393x + 0.001x^2$; ϕ: $y = 0.433 \times (1.015^x) \times (x^{0.874})$; Ψ: $y = 0.288 \times (1.018^x) \times (x^{0.732})$; Ω: $y = 0.714 \times (1.016^x) \times (x^{0.827})$; NV: not valid for this stage.

'Tonatiuh'	Stages of senescence (SPAD values)			
	G (56–43)	S1 (42–29)	S2 (28–15)	S3 (ω) (14–2)
Representative leaf				
CI calculated with the Polynomial function (ρ)	–16.97 to –12.84	–12.53 to –7.98	–7.64 to –2.67	–2.3 to 1.12
Chl *a* [µg cm^{-2}] calculated with Hoerl function (ϕ)	33.61 to 21.99	21.22 to 12.65	12.09 to 5.77	5.36 to 0.82
Chl *b* [µg cm^{-2}] calculated with Hoerl function (Ψ)	NV	9.39 to 5.68	5.44 to 2.73	2.55 to 0.50
Total Chl [µg cm^{-2}] calculated with Hoerl function (Ω)	48.47 to 31.70	30.60 to 18.32	17.52 to 8.51	7.91 to 1.31

species, it is necessary to identify the specific calibration function as was observed in the present research for each cultivar of *Alstroemeria* sp., 'Tonatiuh' and 'Fogo'.

The results obtained with the mean comparison test (*Student*'s *t*-test) between the estimated Chl values with SPAD and the chemical measurements were not coherent with the calculated Δ at the stages of senescence studied. This can be explained because the Δ only considered mean values and not the standard deviation of the data. Therefore, we suggest not to use Δ as a criterion to select the SPAD calibration function for Chl estimation.

The increment in Δ percentages at the end of senescence (S3) could be associated with the changes in tissue turgor and structure of the leaves during aging (Munné-Bosch and Peñuelas 2003) which could modify the transmittance obtained at 942 nm (Süß *et al.* 2015).

It is known that the absorption maxima of extracted pigment strongly depend on the type of solvent and to some degree, on the type of spectrophotometer used (Lichtenthaler and Buschmann 2001). For example, with increasing polarity of the solvent, the red absorption maximum of Chl *a* shifts from 660 to 665 nm, and the blue absorption maximum from 428 to 432 nm. The same applies to Chl *b*, which shifts from 642 to 652 nm and 452 to 469 nm (Lichtenthaler 1984). In our case, the pigment extraction was performed in 80% acetone, where

Table 6. Utility of the calibration functions for *SPAD-502* meter values and color index (CI) in four stages of senescence (G – green, S1 – early senescence, S2 – intermediate senescence, S3 – advanced senescence) of *Alstroemeria* 'Fogo'. ω: the lower *SPAD-502* meter value was 2 considering the precision (± 1) of the instrument; ρ: $y = -0.004 \times (0.962^x) \times (x^{2.612})$; ϕ: $y = 0.124 \times (1.000^x) \times (x^{1.392})$; Ψ: $y = 0.167 \times (1.011^x) \times (x^{0.977})$; Ω: $y = 0.262 \times (1.004^x) \times (x^{1.251})$; NV: not valid for this stage.

'Fogo'	Stages of senescence (SPAD values)			
	G (56–43)	S1 (42–29)	S2 (28–15)	S3 (ω) (14–2)
Representative leaf				
CI calculated with Hoerl function (ρ)	–16.83 to –13.66	–13.33 to –7.70	–7.25 to –1.66	–1.37 to –0.02
Chl *a* [μg cm^{-2}] calculated with Hoerl function (ϕ)	33.64 to 23.29	22.54 to 13.46	12.82 to 5.38	4.88 to 0.33
Chl *b* [μg cm^{-2}] calculated with Hoerl function (Ψ)	NV	NV	5.88 to 2.77	2.57 to 0.34
Total Chl [μg cm^{-2}] calculated with Hoerl function (Ω)	50.39 to 34.38	33.25 to 19.86	18.93 to 8.23	7.52 to 0.63

the absorption maximum for Chl *a* is around 663 nm and 643 nm for Chl *b* (Lichtenthaler 1984). According to the validation of the calibration functions for *SPAD-502* meter, they were more reliable for Chl *a* and total Chl, for both cultivars at all stages of senescence. This can be explained considering that the *SPAD-502* meter calculates a numerical value by division of light transmission intensity at 660 nm by 942 nm (Süß *et al.* 2015).

SPAD values have been extensively used for the estimation of total Chl (Li *et al.* 2017, Donnelly *et al.* 2020). In the case of senescence, pigment degradation starts with the conversion of Chl *b* into Chl *a* (Kuai *et al.* 2018). Later, the Mg^{2+} and the phytol fragment are separated from Chl *a*, and the tetrapyrrole ring becomes linear to produce the fluorescent catabolites to be exported to the vacuole (Matile *et al.* 1996, Kuai *et al.* 2018). The results of the present research indicate that the use of the *SPAD-502* meter for senescence studies is recommended since this instrument estimates Chl *a* in a very accurate manner.

SPAD values can be used for estimating the concentration of Chls, but they cannot be associated with a color. In the present research, we studied the relationship between SPAD values and the CI calculated from CIE L*a*b* system data during foliar senescence of *Alstroemeria.* We observed a much stronger fit using a second-order Polynomial function for both cultivars. With this function, it is possible to estimate a SPAD value from a color index and *vice versa*. It is important to mention that the adjustment functions for both SPAD values and Chls, as well as those related to color, must be genotype-specific and cannot be generalized because each species and cultivar have morphological, biochemical, and color characteristics. This demonstrated that color measurements can be used to estimate the Chl content instead of the *SPAD-502* meter, particularly in laboratories where color measurements are performed routinely.

Conclusions: According to our results, the *SPAD-502* meter is a reliable instrument to measure Chl *a* and total Chl. For the proper selection of a calibration function, it is necessary to take into consideration the following criteria: (*1*) parsimonious, with few parameters, (*2*) an R^2 value closer to one, and (*3*) the lowest AIC. We found that it is important to corroborate the selected function by performing a statistical comparison between the Chl values estimated from SPAD values and their chemical Chl concentrations. In a model of foliar senescence, we validated that SPAD values converted from CI can be easily associated with four stages of foliar senescence (G, S1, S2, S3), in two cultivars of *Alstroemeria* sp., 'Tonatiuh' and 'Fogo'. In the present study, we demonstrated that the CIE L*a*b* system, based on an optic geometry, and the *SPAD-502* meter, based on a transmittance system, both

are handy and reliable methods to monitor leaf Chl, in a nondestructive manner, without toxic chemicals.

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