



Nondestructive analysis of photosynthetic pigments in forage radish and vetch

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ABSTRACT

Chlorophylls and carotenoids are the main photosynthetic pigments in plants, and photosynthetic potential can be used to determine the nitrogen fixation or cycling capacity of cover crops. Therefore, this study quantified and compared the content of photosynthetic pigments in *Raphanus sativus* and *Vicia sativa* by using two different methods, namely the extraction method and measurements with a portable chlorophyll meter, and determined whether the results of these two methods showed a significant correlation. Photosynthetic pigments were measured, using both methods, in a greenhouse and laboratory, at four developmental stages: the vegetative stages I (30 days after sowing [DAS]), II (60DAS), and III (90DAS), and the reproductive stage (150DAS). The determination of the photosynthetic pigment content in *R. sativus* and *V. sativa*, obtained from the extraction method and chlorophyll index, revealed significant differences depending on the developmental stage in both species. Furthermore, the contents of chlorophyll a, chlorophyll b, and carotenoids determined using the extraction method showed high coefficients of correlation with the total chlorophyll index determined using the portable chlorophyll meter. Therefore, the portable chlorophyll meter can be used for the accurate evaluation of the photosynthetic potential of *Raphanus sativus* and *Vicia sativa*, which also saves time and reagents.

Keywords: *Raphanus sativus* L.; *Vicia sativa* L.; carotenoids; chlorophyll; portable chlorophyll.

INTRODUCTION

The forage radish (*Raphanus sativus* L.) and vetch (*Vicia sativa* L.) are forage species considered important for cultivation during winter in southern Brazil, especially to cover and protect soil with plants, plant residues in no-tillage systems, and as weed suppressors (Cutti *et al.*, 2016; Krenchinski *et al.*, 2018). The forage radish is mainly used due to its fast growth, low nutrient demand, high tolerance to climatic adversities, and pivotal root system, which increases soil exploration and may improve soil physical conditions, in addition to presenting an intense nutrient cycling rate, with easy availability to the crop in succession (Cardoso *et al.*, 2014; Krenchinski *et al.*, 2018). As for the vetch, it is widely used because of its considerable nitrogen fixation capacity and rapid

availability to next crops, which is illustrated by the fact that it can provide more than 200 kg of nitrogen per hectare (Restovich *et al.*, 2012).

In current agriculture, the photosynthetic efficiency of crop plants is used to determine the rate of nitrogen fertilization or the potential amount of nitrogen that cover crops can bring to the next crop because the total chlorophyll content is associated with the organic nitrogen content of leaves and, consequently, crop yield in many crops (Smeal & Zhang, 1994; Rigon *et al.*, 2012; De Castro *et al.*, 2014; Cavalcante *et al.*, 2016; Kaspary *et al.*, 2019).

The content of photosynthetic pigments are measured mostly using the traditional methodology, which implies the destruction of leaf samples. This procedure is costly as it involves the use of laboratory reagents, and it is impractical because it does not produce instant results.

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In contrast, portable chlorophyll meters rely on nondestructive, simple, and instantaneous measurements (Salla *et al.*, 2007). In recent years, portable chlorophyll meters have been successfully used to measure the content of photosynthetic pigments and leaf nitrogen content in different species (Ciganda *et al.*, 2009; Rigon *et al.*, 2012; Rigon *et al.*, 2013; Kaspary *et al.*, 2014b; Kaspary *et al.*, 2019).

Chlorophyll meters indirectly determine the relative chlorophyll content, which is calculated from the amount of light transmitted by the leaf, measured at wavelengths with variable absorbance, hence providing a unique reading that is proportional to the contents of chlorophyll a, chlorophyll b, and carotenoids (Minolta Camera Company, 1989; Falker Automação Agrícola, 2009). Models of chlorophyll meters describe the relationship between the readings displayed by the portable chlorophyll meter and extractable chlorophyll according to species and their intrinsic characteristics, which requires independent calibration (Lee, 1988; Markwell *et al.*, 1995; Uddling *et al.*, 2007). To date, no studies have been conducted to understand the relationship between different methods for the measurement of photosynthetic pigments in *R. sativus* and *V. sativa*.

The objective of the present study was to quantify and compare the content of photosynthetic pigments in the cover crops *Raphanus sativus* and *Vicia sativa* using two different methods, namely the extraction method and measurements with a portable chlorophyll meter, and to determine whether the results of these methods were significantly correlated.

MATERIALS AND METHODS

Plant material

This study was conducted in a greenhouse and in the Laboratory of Chemical Analysis of the Department of Agronomic and Environmental Sciences of the Universidade Federal de Santa Maria (UFSM), Campus of Frederico Westphalen, RS - Brazil, from May 2013 to November 2013. Seeds of *R. sativus* and *V. sativa* were sown in 6 L plastic pots filled with agricultural substrate. After seed germination, only one seedling was retained per pot. Photosynthetic pigments were measured, using both methods, at four developmental stages: the vegetative stages I (30 days after sowing [DAS]), II (60 DAS), and III (90 DAS), and the reproductive stage (150 DAS). To measure the photosynthetic pigments at each developmental stage, two leaves from the middle third of the main stalk of the same plant were analyzed and collected. For each species, this procedure was repeated with four plants, totaling eight replicates per treatment.

Estimation of pigment content

The relative chlorophyll content was measured individually using a ClorofiLOG1030® (Falker, Porto Alegre, Brazil) portable meter, and the index reading was obtained directly from the leaf without removing it from the plant. The relative chlorophyll content determined using the portable chlorophyll meter was calculated from the amount of light transmitted by the leaf, based on the wavelength with variable absorbance, thus instantaneously providing a single reading proportional to the content of chlorophyll a, chlorophyll b, and carotenoids (Rigon *et al.*, 2012). Notably, the portable ClorofiLOG1030 meter operates at the following three wavelengths: 635, 660, and 850 nm (Rigon *et al.*, 2012).

Measurement of chlorophyll using a destructive method

Leaves of *R. sativus* and *V. sativa* were collected and taken to the laboratory for analysis with the extraction methodology, as described previously (Hiscox & Israelstam, 1979). To dissolve the samples, 0.05 g of fresh leaf weight (FLW) was placed in test tubes containing 3 mL of dimethyl sulfoxide (DMSO) and incubated in a water bath at 65 °C for 45 min. Subsequently, 2 mL of DMSO was added, and samples were manually shaken for 30 s. Then, the absorbance of the final solution (2.5 mL) containing the pigments was measured using a BioMate3 spectrophotometer (ThermoFischer, Madison, WI, USA) at 470, 645, and 663 nm for carotenoids, chlorophyll b, and chlorophyll a, respectively. Readings obtained at each wavelength were used in the equations reported by Lichtenthaler (1987), based on the FLW, to determine the content of total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids, in mg g⁻¹.

Statistical analysis

The data obtained for *R. sativus* and *V. sativa* using the two methods were subjected to analysis of variance using the F test, and the means of the treatments were compared using the DMS test, with a 5% probability level. The correlation between the readings obtained using the extraction method and with the portable chlorophyll meter was assessed with a regression analysis. Curve adjustments were performed with the quadratic function, according to the determination coefficient, using Sigmaplot® version 11.2.

RESULTS

Nondestructive method of chlorophyll measurement

The results obtained in the present work demonstrated that the chlorophyll content measured with the portable chlorophyll meter varied significantly with the

developmental stage of *R. sativus* and *V. sativa* ($P \geq 0.05$ for both; Table 1). For *R. sativus*, the chlorophyll index measured at the vegetative stage I was the lowest (i.e., 43.0), whereas that measured at the vegetative stage II was the highest (i.e., 49.6). As for *V. sativa*, the mean chlorophyll index measured at the vegetative stage III was the highest (i.e., 50.4), whereas that measured at the reproductive stage was the lowest (i.e., 40.1). Notably, the chlorophyll index measured during the reproductive stage of *R. sativus* was intermediate between the indices measured during other stages, with a value of 47.2.

Destructive method of chlorophyll measurement in the laboratory

The evaluation of the photosynthetic pigments in *R. sativus* and *V. sativa* with the extraction method revealed that the contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids varied with the developmental stage of the plants (Table 1). For *R. sativus*, these contents were the lowest at the reproductive stage (1.0358, 0.2456, and 2814 mg g⁻¹ FLW for chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids, respectively) and highest at the vegetative stage III (1.4603, 0.2949, 1.8552, and 0.4487 mg g⁻¹ FLW for chlorophyll a, chlorophyll b, and total chlorophyll, respectively; Table 1). At the other stages, the values of these pigments were intermediate, with the exception of carotenoids, whose values did not differ between the vegetative stages I and II and the reproductive stage.

For *V. sativa*, the contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids at the vegetative stage III were 1.9958, 0.3025, 2.2983, and 0.4705 mg g⁻¹ FLW, respectively, thus revealing superior photosynthetic performance at this stage (Table 1). The values of these pigments were intermediate during the vegetative stage I and reproductive stage. These data indicate a greater accumulation of photosynthetic pigments in *R. sativus* than in *V. sativa*.

Comparison between the destructive and nondestructive methods of chlorophyll measurement

The regression analysis of the results obtained using two different methodologies (i.e., measurements with the portable chlorophyll meter and the laboratory extraction method) showed a high correlation, with all determination coefficients above 0.75 (Figures 1 and 2). The correlation between the chlorophyll index for chlorophyll a generated with the chlorophyll meter and that measured with the laboratory extraction method was high, with determination coefficients of 0.85 and 0.91 for *R. sativus* and *V. sativa*, respectively (Figures 1A and 1B). For chlorophyll b, the data showed a quadratic behavior and determination

coefficients of 0.90 and 0.92 for forage radish and vetch, respectively (Figures 1C and 1D). Finally, the chlorophyll index obtained with the chlorophyll meter showed a high capacity to indirectly measure the total chlorophyll content in both species, with determination coefficients of 0.90 and 0.93 for *R. sativus* and *V. sativa*, respectively (Figures 1E and 1F).

The measurement of carotenoids using the laboratory extraction method was strongly correlated with the chlorophyll index obtained with the chlorophyll meter, with determination coefficients of 0.94 and 0.90 for *R. sativus* and *V. sativa*, respectively (Figures 2A and 2B). Based on the high determination coefficients, the chlorophyll a and b contents could be correlated with the indices determined using the chlorophyll meter, and a highly reliable mathematical model was developed for these variables, when analyzed in *R. sativus* and *V. sativa* (Figures 2C and 2D).

DISCUSSION

The results indicated that the chlorophyll index increased in the vegetative stage III for both *R. sativus* and *V. sativa*, before subsequently decreasing in the reproductive stage (Table 1). A higher photosynthetic pigment content facilitates the use of luminosity to increase the rate of liquid accumulation of photoassimilates, which is also linked to a high vegetative growth rate (Fleck *et al.*, 2003). This suggests that forage radish and vetch present a similar capacity to utilize luminosity in the vegetative stages I, II, and III, thus increasing the accumulation of dry matter as well as their competitive potential.

The results of the measurement of photosynthetic pigments using the laboratory extraction method corroborated the results obtained with the chlorophyll meter (Table 1). Notably, such correspondence has also been reported in studies that compared the ClorofiLOG index and laboratory extraction with *Sesamum indicum*, *Ricinus communis*, *Conyza bonariensis*, *Avena strigosa*, and *Avena sativa* (Rigon *et al.*, 2012; Rigon *et al.*, 2013; Kaspary *et al.*, 2014a; Kaspary *et al.*, 2019).

High correlations were observed between the two measurement methods for all the analyzed photosynthetic pigments (Figures 1 and 2). For instance, the correlation for chlorophyll a was high in the present study, with a determination coefficient of 0.91 for *R. sativus* and *V. sativa*, which is similar to the results of previous studies on *L. multiflorum* and *A. strigosa* that obtained determination coefficients of 0.98, 0.96, and 0.91, respectively (Kaspary *et al.*, 2014b; Kaspary *et al.*, 2019). Thus, we conclude that it is possible to efficiently estimate the chlorophyll a content using portable chlorophyll meters and mathematical models that have been developed in parallel

with the laboratory extraction method (Kaspary *et al.*, 2019). In other words, it is an important tool to assess the photosynthetic potential of these species and to infer their nutritional status (Coelho *et al.*, 2012). The nutritional status of plants, which is related to nitrogen availability, can be gauged with chlorophyll indices because nitrogen is part of the structure of chlorophyll (Coelho *et al.*, 2010). In tomatoes, for example, a study showed that the chlorophyll index varied proportionally to the different rates of nitrogen fertilization that were used (Coelho *et al.*, 2010). Therefore, the photosynthetic capacity of the plant as well as its potential for accumulation of photoassimilates and development, can be inferred from the chlorophyll index.

High determination coefficients for chlorophyll b (>0.90 for forage radish and vetch) between the chlorophyll index and the results of the laboratory extraction method corroborate those previously observed in *C. bonariensis*

(>0.90 in the two evaluated biotypes; Kaspary *et al.*, 2014a). Notably, with chlorophyll meters, measuring the chlorophyll b content is more difficult than measuring the chlorophyll a content (Neves *et al.*, 2005) because the wavelength emitted by the apparatus is closer to the absorption peak of chlorophyll a (i.e., 660 nm), whereas the absorption of chlorophyll b has two peaks (i.e., 500 and 650 nm; Di Vittorio, 2009). However, in the present study, high efficiency was observed in the ClorofiLOG readings, in relation to the laboratory analyses, for *R. sativus* and *V. sativa*. Similarly, high reliability coefficients for chlorophyll b were obtained with ClorofiLOG readings in previous studies on *L. multiflorum*, *A. strigosa*, and *A. sativa* (Kaspary *et al.*, 2014b; Kaspary *et al.*, 2019).

The total chlorophyll content also showed a high correlation with the chlorophyll index, with determination coefficients of 0.90 and 0.92 for *R. sativus* and *V. sativa*, respectively (Figures 1E and 1F). These values are similar

Table 1: Relative chlorophyll index generated by the ClorofiLOG meter, and chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids (FLW mg g⁻¹) obtained with the extraction method, in *R. sativus* and *V. sativa*.

	Chlorophyll index			
	Vegetative - I	Vegetative - II	Vegetative - III	Reproductive
<i>Raphanus sativus</i>	43.0 B ²	49.6 A	51.3 A	47.2 AB
CV (%) ¹			8.93	
<i>Vicia sativa</i>	42.9 B	45.4 AB	50.4 A	40.1 B
CV (%)			7.23	
	Chlorophyll a			
	Vegetative - I	Vegetative - II	Vegetative - III	Reproductive
<i>Raphanus sativus</i>	1.1174 B	1.4273 A	1.4603 A	1.0358 B
CV (%)			5.64	
<i>Vicia sativa</i>	1.1046 B	1.2298 B	1.9958 A	1.0088 B
CV (%)			8.91	
	Chlorophyll b			
	Vegetative - I	Vegetative - II	Vegetative - III	Reproductive
<i>Raphanus sativus</i>	0.2658 AB	0.2623 AB	0.2949 A	0.2456 B
CV (%)			8.04	
<i>Vicia sativa</i>	0.2296 B	0.2036 B	0.3025 A	0.1977 B
CV (%)			8.74	
	Chlorophyll total			
	Vegetative - I	Vegetative - II	Vegetative - III	Reproductive
<i>Raphanus sativus</i>	1.3832 B	1.6896 A	1.8552 A	1.2814 B
CV (%)			12.01	
<i>Vicia sativa</i>	1.3342 B	1.4334 B	2.2983 A	1.2065 B
CV (%)			7.45	
	Carotenoids			
	Vegetative - I	Vegetative - II	Vegetative - III	Reproductive
<i>Raphanus sativus</i>	0.2875 B	0.3647 B	0.4487 A	0.3648 B
CV (%)			10.01	
<i>Vicia sativa</i>	0.3823 B	0.4198 B	0.4705 A	0.3596 C
CV (%)			6.75	

¹Coefficient of variation. ²Similar uppercase letters in the row do not differ from one another by the DMS test at 5% significance.

to those obtained using the correlation method with *Gossypium hirsutum* and *S. indicum*, with coefficients of 0.91 and 0.98, respectively (Brito *et al.*, 2011; Rigon *et al.*, 2012). This confirms that it is possible to adjust a highly

reliable mathematical model for this variable when comparing pigment measurements that were performed, in forage radish and vetch, using different methods (Figures 1E and 1F).

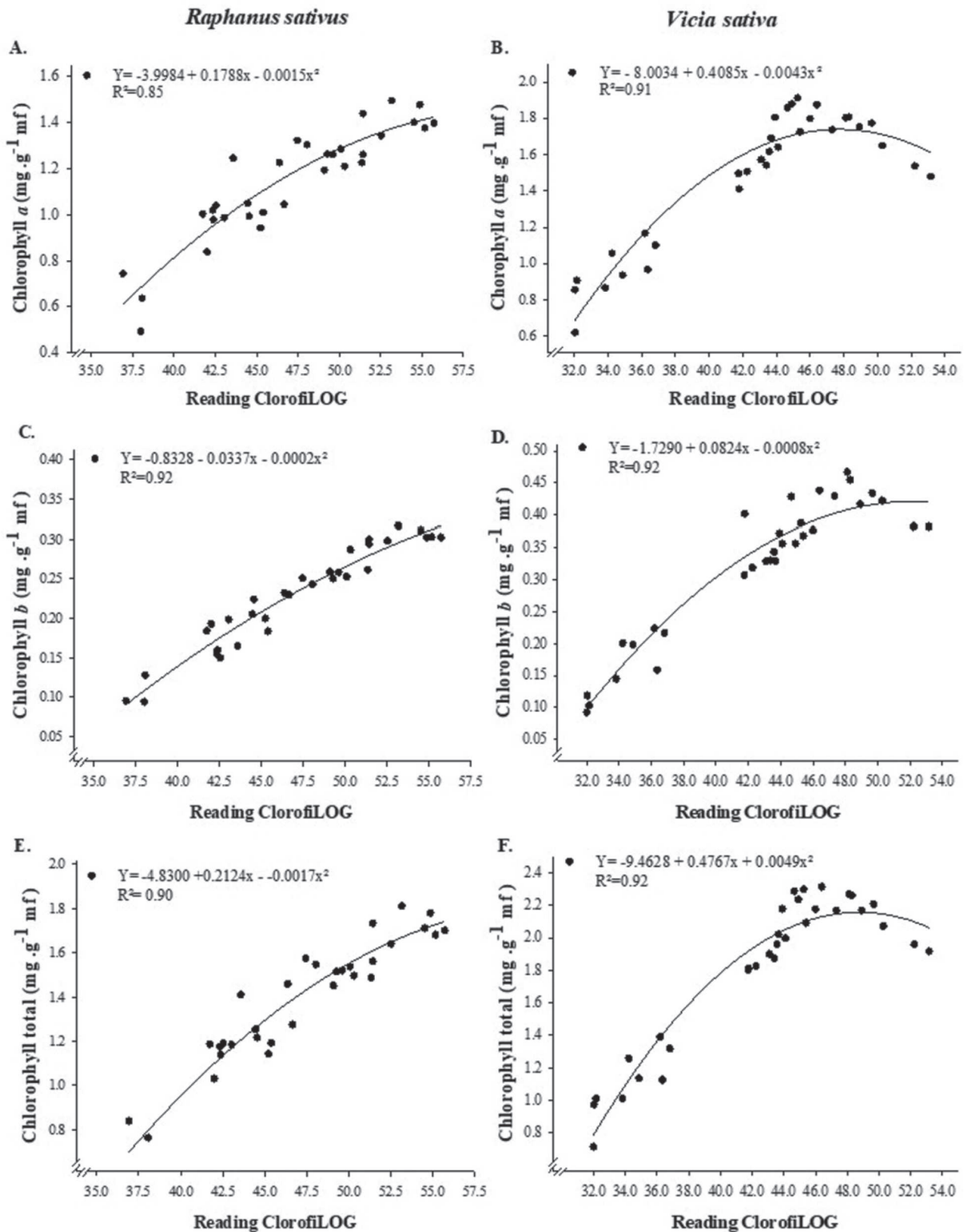


Figure 1: Relationship between the readings of the portable ClorofiLOG for chlorophyll a, chlorophyll b, and total chlorophyll contents in *R. sativus* (A, C, and E) and *V. sativa* (B, D, and F).

The relationship between chlorophyll and carotenoid contents of leaves in *R. sativus* and *V. sativa*, shown in Figures 2A and 2B, present equations of high representativeness ($R^2 > 0.90$ for forage radish and vetch), enabling the estimation of the actual content of these plant pigments from the readings of the ClorofiLOG meter. Notably, similar results have been reported for corn and cotton (Ciganda *et al.*, 2009; Brito *et al.*, 2011). Measuring carotenoids is important because these pigments act as photoprotectors in photosystem II and dissipate excess light energy, and, owing to their high antioxidant potential, they prevent chlorophyll damage (Li *et al.*, 2010). Furthermore, the ratio of chlorophyll to carotenoid is altered under stress conditions, especially under water stress (Young & Britton, 1990). Thus, the rapid and accurate measurement of carotenoid content helps infer the state of the plant. Using reliable models, the carotenoid content in *R. sativus* and *V. sativa* can be

determined from the readings obtained with portable chlorophyll meters.

The correlation analysis between the chlorophyll index measured with the ClorofiLOG meter and the extracted chlorophyll a and b contents measured using the laboratory extraction method revealed high determination coefficients, namely 0.75 and 0.87 for *R. sativus* and *V. sativa*, respectively. The relationship between chlorophyll a and chlorophyll b is important in assessing the ability of plants to capture light under shade conditions (Nakazono *et al.*, 2001). In the present study, the results for the forage radish and vetch are similar to those previously observed for *R. communis*, *A. strigosa*, and *A. sativa*, which were characterized by determination coefficients of 0.70, 0.89, and 0.87, respectively (Rigon *et al.*, 2012; Kaspary *et al.*, 2019).

Overall, the measurement of photosynthetic pigments in *R. sativus* and *V. sativa* using a portable chlorophyll

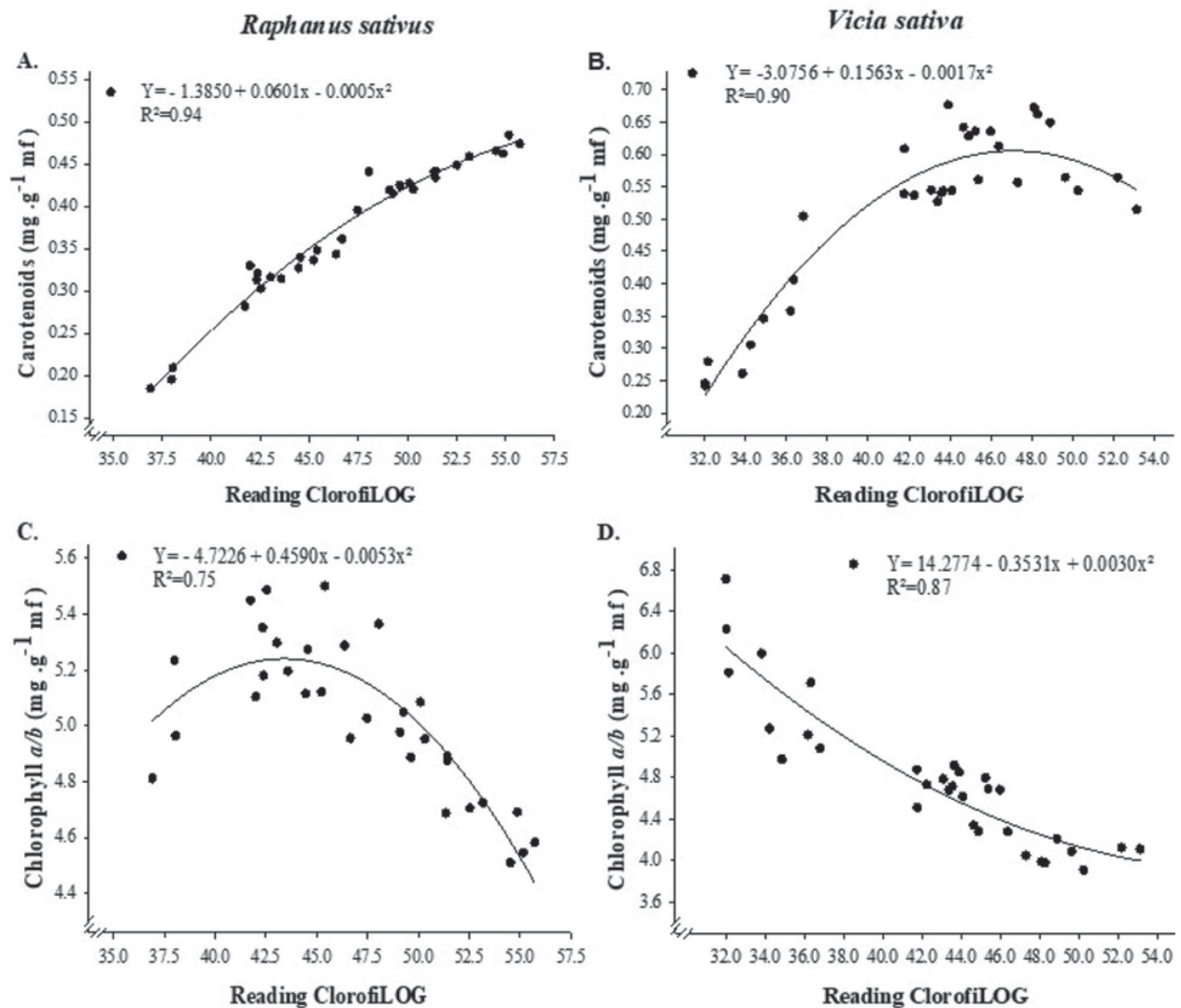


Figure 2: Relationship between the readings of the portable ClorofiLOG for carotenoid contents and chlorophyll a/b ratio in *R. sativus* (A and C) and *V. sativa* (B and D).

meter and adjusted mathematical models is accurate, efficient, and economical in comparison to the laboratory extraction method. Thus, the chlorophyll meter is an important tool to determine the photosynthetic potential of forage radish and vetch.

CONCLUSION

The determination of the photosynthetic pigment content in *R. sativus* and *V. sativa* with the extraction method and chlorophyll index revealed significant differences in pigment levels depending on the developmental stage, in both species.

The measurement of the photosynthetic pigments in *R. sativus* and *V. sativa* using a portable chlorophyll meter and adjusted mathematical models is accurate and saves time and reagents compared to the laboratory extraction method.

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