Scientia Agricola
http://dx.doi.org/10.1590/1678-992X-2015-0477

Estimating foliar nitrogen in *Eucalyptus* using vegetation indexes

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Edited by: Rafael Rubilar Pons

Received December 10, 2015
Accepted June 02, 2016

ABSTRACT: Nitrogen (N) has commonly been applied in *Eucalyptus* stands in Brazil and it has a direct relation with biomass production and chlorophyll content. Foliar N concentrations are used to diagnose soil and plant fertility levels and to develop N fertilizer application rates. Normally, foliar N is obtained using destructive methods, but indirect analyses using Vegetation Indexes (VIs) may be possible. The aim of this work was to evaluate VIs to estimate foliar N concentration in three *Eucalyptus* clones. Lower crown leaves of three clonal *Eucalyptus* plantations (25 months old) were classified into five color patterns using the Munsell Plant Tissue Color Chart. For each color, N concentration was determined by the Kjeldahl method and foliar reflectance was measured using a CI-710 Miniature Leaf Spectrometer. Foliar reflectance data were used to obtain the VIs and the VIs were used to estimate N concentrations. In the visible region, the relationship between N concentration and reflectance percentage was negative. The highest correlations between VIs and N concentrations were obtained by the *Inflection Point Position* (IPP, r = 0.97), *Normalized Difference Red-Edge* (reNDVI, r = 0.97) and *Modified Red-Edge Normalized Difference Vegetation Index* (mNDI, r = 0.97). Vegetation indexes on the red edge region provided the most accurate estimates of foliar N concentration. The reNDVI index provided the best N concentration estimates in leaves of different colors of *Eucalyptus urophylla* × *grandis* and *Eucalyptus urophylla* × *urophylla* (R^2 = 0.97 and RMSE = 0.91 g kg^{-1}).

Keywords: leaf nitrogen concentration, non-destructive analyses, spectrometer, spectral reflectance, leaf reflectance

Introduction

Nitrogen (N) is one of the essential elements required in many sites to optimize growth of *Eucalyptus* stands in Brazil (Santana et al., 2000). Nitrogen is associated with several important proteins in the photosynthetic process (Moran et al., 2000; Ollinger, 2011). Therefore, it has a direct relationship with chlorophyll (Schlemmer et al., 2013) and biomass production (Miller, 1984; Smethurst et al., 2004).

Foliar N analyses are commonly obtained using destructive sampling methods and little progress has been made in the development of indirect analyses based on spectral characteristics (Ollinger, 2011). In this context, the use of non-destructive analyses using the foliar reflectance spectrum to estimate the chlorophyll content has been improved (Gitelson et al., 2009; Sims and Gamon, 2002; Ustin et al., 2009). The reduction of chlorophyll content implies lower energy absorption in the visible spectrum (400 – 700 nm) and consequently greater foliar reflectance in the electromagnetic spectrum (Richardson et al., 2002).

Chlorophyll has two absorption peaks of electromagnetic energy in the visible region, one in the blue (400 – 500 nm) and another in the red region (620 – 700 nm) (Lichtenthaler, 1987). Therefore, these regions are commonly used to make chlorophyll estimations. Still, in the blue region, energy absorption can occur from other foliar pigments that might significantly hinder estimation of the chlorophyll content for these wavelengths (Buschmann et al., 1994). Consequently, the best estimates of the chlorophyll content are wavelengths in the green (500 – 570 nm) and red regions, in addition to the region known as the red edge (700 – 750 nm) (Clevers and Gitelson, 2013; Gitelson et al., 2003; Richardson et al., 2002; Ustin et al., 2009).

According to these concepts, several authors have developed Vegetation Indexes (VIs) that provide an accurate estimation of the leaf chlorophyll content (Ustin et al., 2009) in several species, including the *Eucalyptus* (Datt, 1998, 1999a,b; Sims and Gamon, 2002). Based on the close relationship between chlorophyll and N, these VIs have been used to estimate N concentration in rice (Tian et al., 2011; Yao et al., 2013), maize (Schlemmer et al., 2013), grasses and potatoes (Clevers and Gitelson, 2013). However, the use of such indexes to estimate N concentration in *Eucalyptus* is rare in the literature. Therefore, the aim of this work was to evaluate vegetation indexes to estimate foliar N concentration in three *Eucalyptus* clones.

Materials and Methods

The study was conducted in the municipality of Lassance, Minas Gerais, Brazil, within a 30 km radius from the centroid 17°56,647' S, 44°52,710' W and at about 800 m a.s.l. (Gerdau Florestal SA plantations). Leaf sampling was conducted in Apr 2014 using commercial plantations of clonal *Eucalyptus* at 25 months old and established at a 7.0 × 1.3 m tree spacing. The soil at the experimental location was characterized as an Oxisol (Typic Hapludox) with a sandy loam texture.

We used three *Eucalyptus* clones that are widely grown in Minas Gerais State, Brazil (GG680: 40000 ha, GG682: 2700 ha, I144: 17200 ha). Lower crown leaves were classified into five color patterns using the Munsell Plant Tissue Color Chart (Table 1). This region was chosen due to the high N cycling, which results in a more variable N concentration in leaves (Saur et al., 2000). In the field, 30 leaves of each color pattern were randomly collected from the lower crown in three 10-ha plots in plantations of three different *Eucalyptus* clones (Table 1). Immediately after collecting each leaf, readings of foliar reflectance were made (400 – 900 nm). The readings were consistently made on the left side of the leaves at 10 mm from the lower border using a CI-710 Miniature Leaf Spectrometer (CID Bio-Science). The leaves were oven-dried at 65 °C until constant weight, milled (mesh size 1 mm) and N concentrations measured using the Kjeldahl method (AOAC, 1990).

Foliar reflectance was analyzed using SpectraSnap! (Software version 1.1.3.150, CID Bio-Science), with 300 milliseconds of integration time, boxcar width of 10 points and 2 scans to average. Subsequently, the reflectance spectrums were smoothed using the Savitsky-Golay algorithm (Savitzky and Golay, 1964) with a second-degree polynomial model. The smoothed spectrums were used to obtain the Vegetation Indexes (VIs) and the VIs were used to estimate foliar N concentrations (Table 2).

Relations between VIs and N concentration were evaluated by the Pearson correlations (r), and their significance for statistic "t" ($p < 0.01$). In cases of significant r, they were adjusted using exponential, simple linear, logarithmic and quadratic regression equations. VIs were used as the independent variable and N concentration as the dependent variable. The equations chosen were those that best adapted to each index, evaluated by the coefficient of determination (R^2) and Root Mean Square Error (RMSE) (1). All procedures were carried out using the software R Core Team (2015) 3.1.3 version, platform version support R Studio 0.98.1103.

$$RMSE\left(g\ kg^{-1}\right) = \sqrt{\frac{\sum_{i=1}^{n}\left(Yi - yi\right)^{2}}{n}}$$

where: Yi is the N concentration of the i sample estimated by the equation; yi is the N concentration observed in the lab for sample i; and n is the total number of samples.

Table 1 – Number of leaves sampled by color patterns (Munsell Plant Tissue Color Chart) and genetic material.

Eucalyptus clone	Name	Plot	Area	7.5 GY 8/8 🟩	7.5 GY 8/4 🟩	7.5 YR 4/2 🟫	2.5 Y 7/6 🟨	2.5 Y 8/10 🟨
			ha			number of leaves sampled		
		1	10	30	30	30	30	30
E. urophylla × *E.grandis*	GG680	2	10	30	30	30	30	30
		3	10	30	30	30	30	30
		1	10	30	30	30	30	30
E. urophylla × *E. grandis*	GG682	2	10	30	30	30	30	30
		3	10	30	30	30	30	30
		1	10	30	30	30	30	30
E. urophylla × *E. urophylla*	I144	2	10	30	30	30	30	30
		3	10	30	30	30	30	30

Table 2 – Vegetation indexes used to estimate foliar nitrogen concentrations.

Vegetation Indexes	Short	Formula	Source
Normalized Difference Vegetation Index	NDVI	$(\rho_{NIR} - \rho_{R})/(\rho_{NIR} + \rho_{R})$	Tucker (1979)
*Red-edge Inflexion Point Position**	IPP	$\rho'(\lambda_{i})=0$	Horler et al. (1983)
Normalized Difference Red-Edge	reNDVI	$(\rho_{750} - \rho_{705})/(\rho_{750} + \rho_{705})$	Gitelson and Merzlyak (1994)
Structure Insensitive Pigment Index	SIPI	$(\rho_{800} - \rho_{445})/(\rho_{800} - \rho_{680})$	Peñuelas et al. (1995)
Green Normalized Difference Vegetation Index	gNDVI	$(\rho_{NIR} - \rho_{G})/(\rho_{NIR} + \rho_{G})$	Gitelson et al. (1996)
Photochemical Reflectance Index	PRI	$(\rho_{531} - \rho_{570})/(\rho_{531} + \rho_{570})$	Gamon et al. (1997)
Eucalyptus Pigment Index	EPI	$\rho_{672}/(\rho_{550} \times \rho_{708})$	Datt (1998)
New Reflectance Chlorophyll Index	NRIC	$(\rho_{850} - {}_{710})/(\rho_{850} - \rho_{680})$	Datt (1999a)
First Derivative	D1	$(\rho'_{754}/\rho'_{704})$	Datt (1999b)
Reciprocal Reflectance	RR	ρ_{700}^{-1}	Gitelson et al. (1999)
Modified Red-Edge Normalized Difference Vegetation Index Modified	mNDI	$(\rho_{750} - \rho_{705})/(\rho_{750} + \rho_{705} - 2\times\rho_{445})$	Sims and Gamon (2002)
Green Chlorophyll Index	CI_{Green}	$(\rho_{NIR}/\rho_{G}) - 1$	Gitelson et al. (2003)
Red-edge Chlorophyll Index	$CI_{RedEdge}$	$(\rho_{NIR}/\rho_{Red\ Edge}) - 1$	Gitelson et al. (2003)

*obtained by first derivative of reflectance; ρ = Reflectance; NIR = Near infrared; R = Red region; G = Green region.

Results

The different leaf colors provided different reflectance spectrum. In general, in the visible region, the relationship between N concentration and reflectance percentage was negative. The lower reflectance percentage implied greater N concentration (Figure 1). On average, the 7.5 GY 8/8 leaves had the lowest intensity of reflectance in nearly all visible regions (440 – 700 nm). In the transition between the visible and near infrared (NIR) region, this color had greater intensity of reflectance (740 – 900 nm). The 7.5 GY 8/4 leaves showed a similar behavior spectrum to the previous color, differing by only a greater percentage of reflectance in the visible region. Different from the others, the 7.5 YR 4/2 leaves exhibited reflectance with a more linear tendency. Common to all leaf colors, an abrupt drop in reflectance occurred in the 655 – 690 nm region. A reversal in reflectance trends between the 7.5 YR 4/2 and 2.5 Y 7/6 leaves was also apparent in this region (shaded region, Figure 1). This change in the spectral reflectance was contrary to the inverse relationship between N concentration and reflectance percentage for this region. The reflectance behavior of the 2.5 Y 7/6 leaves were similar to that for the 2.5 Y 8/10 leaves, which had the foliar color with the highest reflectance percentage in the visible region.

The largest correlation coefficients (r) between VIs and N concentrations were obtained for the *Red-edge Inflexion Point Position* (IPP), *Normalized Difference Red-Edge* (reNDVI) and *Difference Vegetation Index Modified* (mNDI) indexes (Table 3). The only non-significant correlation was for *First Derivative* (D1). The indexes *Structure Insensitive Pigment Index* (SIPI) and *Eucalyptus Pigment Index* (EPI) were related inversely to foliar N concentrations.

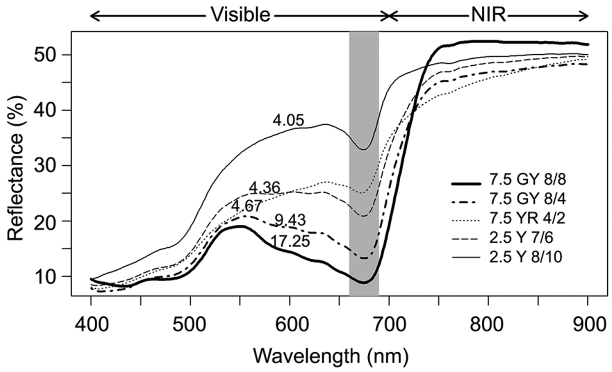

Figure 1 – Average spectral reflectance by foliar color pattern. Each curve represents the average of 270 samples of leaf reflectance. Numbers near the curves indicate the average of N concentration (g kg^{-1}). NIR = Near infrared. Shaded region: reversal in reflectance trends between the 7.5 YR 4/2 and 2.5 Y 7/6 leaves.

The exponential model was well adapted to all VIs, except for IPP, which was best fitted using a simple linear model (Figure 2). The analysis of R^2 and RMSE results showed three regression quality groups. The best group had $R^2 \geq 0.92$ and $RMSE \leq 1.60$ g kg^{-1} (Figures 2A, B, C, D, E, F, G), the good quality group, $0.92 > R^2 > 0.80$ and $2.40 > RMSE > 1.60$ g kg^{-1} (Figures 2H, I), and low quality group, $R^2 \leq 0.80$ and $RMSE \geq 2.40$ g kg^{-1} (Figures 2J, K, L)

Discussion

The green leaves (7.5 GY 8/8) had higher N concentrations and lower reflectance percentages in practically every region of the visible spectrum (Figure 1). Similarly, the results obtained by Ayala-Silva and Beyl (2005) and Yao et al. (2013) showed that lower N concentration provided greater reflectance percentages in the visible region. Nevertheless, in this work, the reflectance trends were reversed for the 7.5 YR 4/2 and 2.5 Y 7/6 leaves in the wavelengths 600 – 685 nm. This reversal may be related to the influence of compounds with other elements, which also absorb energy in this region of the spectrum. Nevertheless, this reversal affected the calculation of VIs that used at least one wavelength in this region such as *New Reflectance Chlorophyll Index* (NRIC), SIPI and EPI (Table 2) and may have compromised the quality of the fit of those indexes (Figures 2J, K, L).

Based on the R^2 and RMSE (Figure 2), the VIs that provided stronger predictions were those that estimated the N concentration in wavelengths of the red edge region (700 – 750 nm) such as reNDVI, mNDI, *Red-edge Chlorophyll Index* ($CI_{Red\ Edge}$) and *Reciprocal Reflectance* (RR), in addition to the *Normalized Difference Vegetation Index* (NDVI) and IPP, which use, respectively, the red band and the position of the highest inflection point of the curve in the region of the red and red edge region (Figure 2). In other studies, the VIs that used these regions generally provided more accurate estimates than those operating in the green region (Clevers and Kooistra, 2012; Inoue et al., 2012; Yao et al., 2013).

Table 3 – Pearson correlation coefficients (r) between vegetation indexes (VIs) and foliar nitrogen concentrations (N).

	IPP	reNDVI	mNDI	$CI_{Red\ Edge}$	RR	NDVI	PRI	gNDVI	CI_{Green}	NRIC	SIPI	EPI	D1
N (g kg^{-1})	0.97*	0.97*	0.97*	0.96*	0.94*	0.93*	0.87*	0.87*	0.87*	0.72*	-0.71*	-0.47*	-0.04^{ns}

*significant by the "t" test ($p < 0.01$); ^{ns}not significant; IPP = *Red-edge Inflexion Point Position*; reNDVI = *Normalized Difference Red-Edge*; mNDI = *Modified Red-Edge Normalized Difference Vegetation Index Modified*; $CI_{Red\ Edge}$ = *Red-edge Chlorophyll Index*; RR = *Reciprocal Reflectance*; NDVI = *Normalized Difference Vegetation Index*; PRI = *Photochemical Reflectance Index*; gNDVI = *Green Normalized Difference Vegetation Index*; CI_{Green} = *Green Chlorophyll Index*; NRIC = *New Reflectance Chlorophyll Index*; SIPI = *Structure Insensitive Pigment Index*; EPI = *Eucalyptus Pigment Index*; D1 = *First Derivative*.

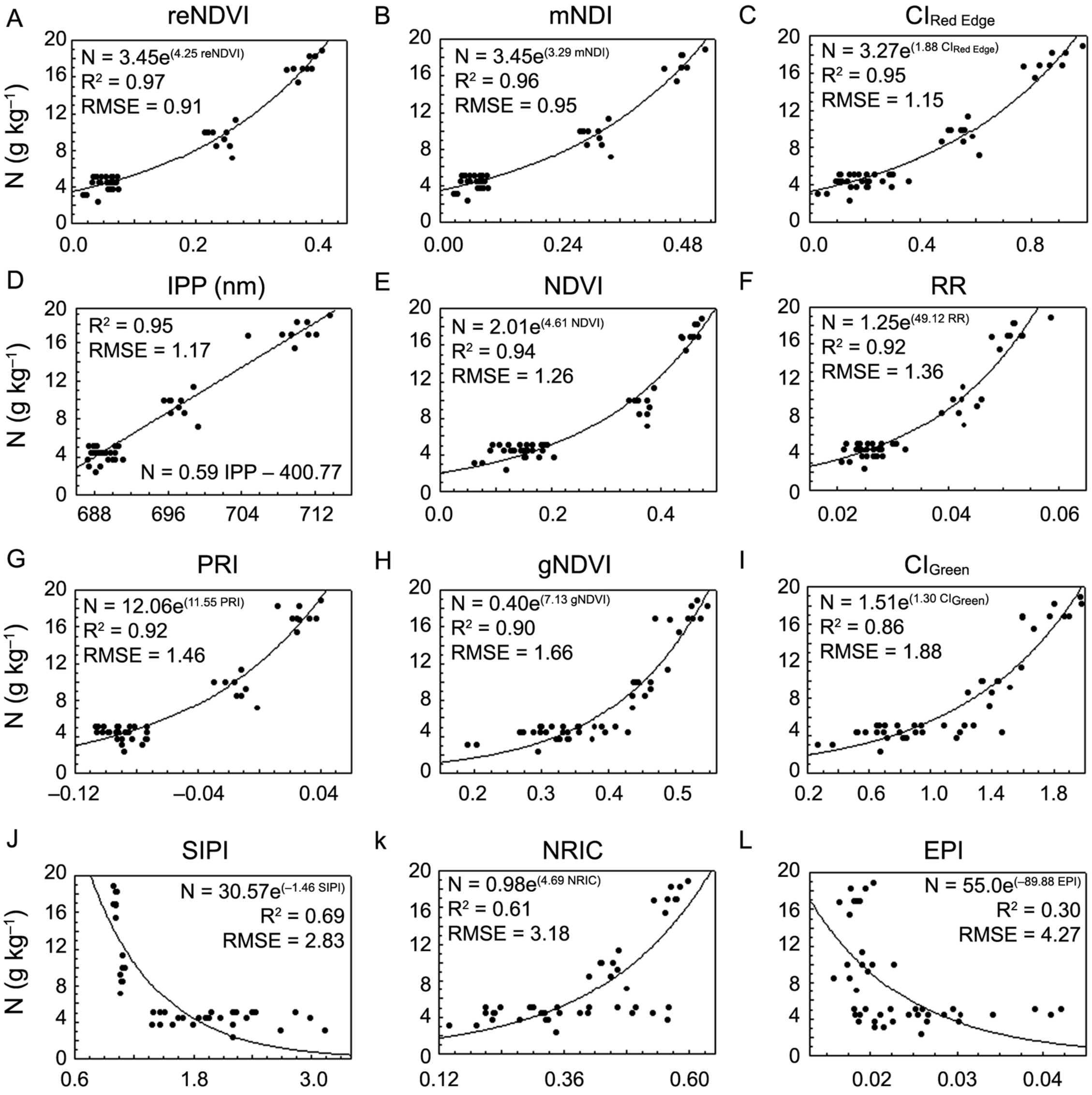

Figure 2 – Adjustments between vegetation indexes and leaf nitrogen concentrations in *Eucalyptus*. RMSE = Root Mean Square Error (g kg^{-1}). IPP = *Red-edge Inflexion Point Position*; reNDVI = *Normalized Difference Red-Edge*; mNDI = *Modified Red-Edge Normalized Difference Vegetation Index Modified*; CI$_{\text{Red Edge}}$ = *Red-edge Chlorophyll Index*; RR = *Reciprocal Reflectance*; NDVI = *Normalized Difference Vegetation Index*; PRI = *Photochemical Reflectance Index*; gNDVI = *Green Normalized Difference Vegetation Index*; CI$_{\text{Green}}$ = *Green Chlorophyll Index*; NRIC = *New Reflectance Chlorophyll Index*; SIPI = *Structure Insensitive Pigment Index*; EPI = *Eucalyptus Pigment Index*.

The NDVI was the first index that was constructed (Tucker, 1979). Since then, the NDVI has been modified to create other indices. These modifications reduced wavelengths used in the calculations (reNDVI and mNDI), added new wavelengths (mNDI) or replaced a region of the electromagnetic spectrum to another (*Green Normalized Difference Vegetation Index*, gNDVI) (Table 2). For the calculation of mNDI, a wavelength was added in the blue region to increase the precision of this index in relation to reNDVI (Sims and Gamon, 2002). In the current study, this modification resulted in a slight reduction in accuracy to estimate N concentration (Figures 2A, B). Most likely, this resulted from saturation of chlorophyll absorption in this region. Low concentrations of chlorophyll are able to saturate absorption in the blue region (Lichtenthaler, 1987), which mainly affected the estimation of higher N concentration (Figures 2A, B). However, for some species using this region in the VIs provided better estimates of N concentrations and chlorophyll contents than reNDVI (Hansen and Schjoerring, 2003; Sims and Gamon, 2002; Tian et al., 2011).

The CI$_{\text{Red Edge}}$ provided good accuracy to estimate N concentrations and it was better than some more used indexes, such as IPP and NDVI (Figures 2C, D, E). Clevers and Gitelson (2013) obtained good results using this index to estimate N concentration in potatoes, as well as Schlemmer et al. (2013) in maize.

For the IPP index (Figure 2D), there was a simple linear relationship between the wavelength and N concentration (Table 2). According to Gates et al. (1965) and Ayala-Silva and Beyl (2005), considering this index, shorter wavelengths indicate lower N concentration in leaves. Buschmann and Nagel (1993), and Mutanga and Skidmore (2007) also states that longer wavelengths indicate higher N concentration in leaves.

The NDVI also provided one of the best N concentration estimates (Figure 2E), even using reversal wavelengths in the reflectance of 7.5 YR 4/2 and 2.5 Y 7/6 color in its calculation (Figure 1). Probably, by using the average value of a range of wavelengths, this variation did not affect the estimation of N as other VIs that used this region. In addition to NDVI, the RR index, composed of only one wavelength, was among the VIs that used the red edge region that provided less accurate estimates of N concentration (Figure 2F). Indexes composed of only one wavelength were less susceptible to variation in leaf N concentrations. Nevertheless, as IPP uses a wavelength of high correlation with N concentration (Clevers and Gitelson, 2013), it provided a good estimate in this study (Figure 2F).

Among the VIs that used the green region in their calculation such as *Photochemical Reflectance Index* (PRI), *Green Chlorophyll Index* (CI_{Green}) and gNDVI, PRI was the only one in the best group for estimation of N concentration (Figures 2G, H, I). Other VIs estimated the N concentration with good quality, but with a high RMSE (> 1.60 g kg^{-1}) (Figures 2G, H, I). The PRI was designed primarily to evaluate its use and effectiveness of photosynthetically active radiation plants for photosynthesis (Gamon et al., 1997). However, as shown in this study, inferences about this index can go beyond that (Figure 2G). The relationship between PRI and N was previously verified in seedlings of *Larix kaempferi* Sarg. (Nakaji et al., 2005). In this study, the gNDVI provided a low estimation accuracy of foliar N if compared to its original index, NDVI (Figures 2E, H). The low accuracy was also noted in the estimation of leaf chlorophyll content for various species of *Eucalyptus*, and provided a less accurate estimate than NDVI (Datt, 1999a). In the green region (500 – 570 nm), chlorophyll had little absorption of light energy (Lichtenthaler, 1987). Similar to *Eucalyptus,* changes in foliar N and chlorophyll contents were not accompanied by the same reflectance variation in this region, which compromised the quality of the adjustments for these VIs (Figures 2G, H, I). However, for leaves of different colors in maple (*Acer platanoides* L.) and chestnut (*Aesculus hippocastanum* L.), gNDVI provided greater sensitivity to the chlorophyll content than NDVI (Gitelson et al., 1996). Among the VIs that used the green region, the CI_{Green} provided less accurate estimations of foliar N concentration (Figure 2I). However, in other species, these indexes present great potential in estimations of N concentration with accuracy similar to or even higher than VIs based on the red edge (Clevers and Gitelson, 2013; Shclemmer et al., 2013).

The other indexes, SIPI, NRIC and EPI, provided low quality estimations of N concentrations (Figures 2J, K, L). Interestingly, some of these were developed especially to estimate chlorophyll content of *Eucalyptus*, like NRIC, EPI and D1 (Datt, 1998, 1999a,b). Differences between the leaf development stage and the plant itself (Gates et al., 1965), time-lapse between measuring reflectance after removing the plant leaf (Gates et al., 1965; Wenjiang et al., 2004) and type of sensor used in the collection of the spectrum reflectance (Erdle et al., 2011) are factors that can change the spectral reflectance of a species and cause differences in the comparison between results even within a species.

Foliar N concentrations in some *Eucalyptus* clones can be successfully predicted using vegetation indexes. The vegetation indexes that were based on the red edge region provided the most accurate estimates of foliar N concentration. The reNDVI was the index that provided better estimates of the N concentration in leaves of different colors of *Eucalyptus urophylla* × *grandis* and *Eucalyptus urophylla* × *urophylla*. We suggest further research to validate the regression equations acquired in other species of *Eucalyptus*.

Acknowledgements

Financial support: Federal University of Jequitinhonha and Mucuri Valleys -UFVJM, Coordination for the Improvement of Higher Level Personnel - CAPES, Brazilian National Council for Scientific and Technological Development - CNPq, Minas Gerais State Foundation for Research Support - FAPEMIG, and Gerdau Florestal SA.

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