Forest Systems
31 (2), e013, 15 pages (2022)
eISSN: 2171-9845
https://doi.org/10.5424/fs/2022312-19242
INIA-CSIC

RESEARCH ARTICLE

OPEN ACCESS

VIS/NIR spectra and color parameters according to leaf age of some *Eucalyptus* species: influence on their classification and discrimination

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Abstract

Aim of study: The aim of this study was to verify the differences in VIS/NIR spectra and leaf color parameters of leaves of *Eucalyptus badjensis, E. benthamii, E. dunnii, E. grandis, E. globulus* and *E. saligna,* at four ages, and their influence on species discrimination.

Area of study: São Mateus do Sul, Paraná, Brazil.

Materials and methods: Seedlings of the six species, with four replicates for each, were acclimatized in the same environment, in October 2015, in an entirely randomized design. Leaf samples were collected from plants that were 6, 8, 10 and 12 months old. Three leaves from each of four plants at each age were analyzed. Five parameters were recorded referring to the adaxial surface of each leaf, with a total of 15 records from repetitions and 60 per species at each age. The evaluation was performed in the spectral ranges from 360-740 nm (VIS) and 1000-2500 nm (NIR). Principal component analysis and linear discriminant analysis were performed.

Main results: The influence of age differed within each species. In color data, the parameter with most variation among all samples was chromatic coordinate b*. In reflectance spectra (VIS), age of 12 months provided the best discrimination of species. Second derivative NIR spectra produced the best results of external prediction of Linear Discriminant Analysis models based on leaves of 12-month-old trees.

Research highlights: Observation of color parameters and VIS/NIR spectroscopy have potential utility for discrimination of Eucalyptus species based on their green leaves.

Additional key words: visible spectra; near infrared spectra; species identification; green leaves; multivariate analysis; chromatic coordinates

Abbreviations used: LDA (linear discriminant analysis); MIR (mid-infrared spectra); NIR (near infrared spectra); PCA (principal component analysis); RENASEM (National Register of Seeds and Seedlings); VIS (visible spectra).

Authors' contributions: Conceptualization: IPM, JM, PVF and SN. Data acquisition and analysis: IPM and SN. Data curation: SN. Supervision JM, PVF, VR, GIBM. Writing and revision: all authors.

Citation: Migacz, IP; Manfron, J; Farago, PV; Raman, V; de Muñiz, GIB; Nisgoski, S (2022). VIS/NIR spectra and color parameters according to leaf age of some *Eucalyptus* species: influence on their classification and discrimination. Forest Systems, Volume 31, Issue 2, e013. https://doi.org/10.5424/fs/2022312-19242

Received: 27 January 2022. **Accepted:** 27 June 2022.

Funding agencies/institutions	Project / Grant
Brazilian National Council for Scientific and Technological Development (CNPq)	PQ 306082/2019-5 for S. Nisgoski

Competing interests: The authors have declared that no competing interests exist.

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Introduction

The presence of over 800 species from genus *Eucalyptus* L'Hér., Myrtaceae family, is registered, most of them native to Australia, but several species are cultivated worldwide for commercial purposes. *Eucalyptus* wood is used in civil construction, the pulp and paper industry, and to produce charcoal for steelmaking, while the leaves are used to extract essential oils. In Brazil, *Eucalyptus* plantations occupy 6.97 million hectares and are located mainly in the states of Minas Gerais, São Paulo and Mato Grosso do Sul (IBÁ, 2020).

The taxonomy of *Eucalyptus* is problematic due to morphological similarities among different species (Steane *et al.,* 2011; Bayly *et al.,* 2013). Visible (VIS) and near infrared (NIR) spectroscopic analysis of leaves has been explored, with adequate results, as a supplementary technique for species identification *Eucalyptus* (Castillo *et al.,* 2008; Abasolo *et al.,* 2013), as well as some Amazonian tree species (Durgante *et al.,* 2013; Lang *et al.,* 2015), among others.

Some recent studies for characterization of *Eucalyptus* species have tested the potential of NIR spectroscopy to predict physical and mechanical properties of *Eucalyptus benthamii* (Estopa *et al.,* 2017), the siringil/guayacil ratio of *E. benthamii* and *Eucalyptus pellita* for use in the pulp and paper industry (Diniz *et al.,* 2019), and to analyze the use of genetic parameters associated with NIR spectroscopy to predict wood quality of an *Eucalyptus urograndis* hybrid (Lima *et al.,* 2019).

Alwi *et al.* (2021) described the use of NIR to predict foliar nitrogen and phosphorus levels of *E. pellita* in the field and also verified the differences in results in function of leaf moisture content, *i.e.,* fresh leaves and dried milled material. Au *et al.* (2020) verified the influence of geographic origin among samples on prediction of nitrogen content, where the results showed that simply relying on spectral variation (assessed by Mahalanobis distance) may mislead researchers about how many reference values are needed.

Using foliage of *Eucalyptus globulus* obtained in common-environment field trials, O'Reilly-Wapstra *et al.* (2013) verified the inheritance pattern in chemical profile and described the use of spectral phenome to differentiate accurately inter-specific hybrids and their parents. Lu *et al.* (2021) tested the potential of NIR to accurately and efficiently discriminate pure species and hybrids within the genus *Eucalyptus* based on fresh and dried leaves, milled leaves of seedlings from pure species *Eucalyptus urophylla* and *Eucalyptus grandis*, and their F1 hybrids.

A review by Cozzolino (2014) described the potential of infrared analysis to detect plant diseases and related the disease incidence with alterations in water, temperature, nutrients, and chemical and physical properties, among other factors, based on plant tissues. NIR spectroscopy can also be used for monitoring contents of water, nitrogen, carbohydrates, protein and oil, among other factors of plants (Jin *et al.,* 2017; Shimbori & Kurata, 2017). Changes in NIR spectra due to leaf age or harvesting period were reported in Coffea taxa leaves (Mees *et al.,* 2018) and for the identification of young and mature trees of species of *Protium* and *Crepidospermum* in the Brazilian Amazon (Lang *et al.,* 2015).

In the visible region, leaf reflectance spectra are influenced by foliar photosynthetic pigments, mainly chlorophyll (Homolová *et al.,* 2013). The 510-520 nm region was associated with carotenoids and bands between 540 and 560 nm were related to anthocyanins (Ustin *et al.,* 2009). Light quality is an important factor that affects tree growth (Lin *et al.,* 2021) and can influence color parameters of leaves (Frizon & Nisgoski, 2020). Some studies have indicated the potential of leaf color for species discrimination (Nisgoski *et al.,* 2015), but they are scarce.

Ballesta *et al.* (2022) described that reflectance spectra from leaves of *Eucalyptus cladocalyx* had some level of genetic control. In particular, the method's based only on spectra were more indicate to predict HCN content in *Eucalyptus* leaves than that applying information with genetic and/or pedigree data. Li *et al.* (2019), evaluating *Sassafras tzumu* leaves, first tested NIR spectroscopy to predict leaf color (parameters a*, b* and L*) and chlorophyll content, after applied results in genetic control and verified that data from fresh leaves in vivo were most efficient in prediction of color and chlorophyll content. On the other hand, da Silva Junior *et al.* (2021), applying hyper and multispectral bands to predict boron levels in *Eucalyptus,* related that region from 425 nm to 475 nm can be applied to identify boron toxicity in plants; adequate boron levels can be observed in spectral ranges from 426 nm to 444 nm, 1811 nm to 1910 nm, 1948 nm to 2115 nm, and 2124 nm to 2208 nm; and boron deficiency was verified in bands from 350 nm to 371 nm.

Studies involving the ages of leaves from *E. grandis* based on VIS, NIR and mid-infrared (MIR) spectroscopy concluded that the response of VIS spectra was different regarding the plant age and diverse directions of sample collection, and that NIR/MIR spectra had no standard reflectance response relating to population ages (Herrera *et al.,* 2017). Silva *et al.* (2021) evaluated *Hevea brasiliensis* leaves, obtained from 14 clones and englobing different tree ages, *i.e.,* young, adult and old trees, and also diseased leaves, based on visible and near infrared spectroscopy. They concluded that the use of spectra obtained from young or old trees, healthy or diseased leaves, enabled the efficient discrimination of rubber trees clones.

The purpose of this research was to verify the differences in near infrared, visible spectra and color parameters of green leaves at four different ages of *Eucalyptus* species and the influence on their discrimination. An additional objective was to contribute information to a database for future practical application in breeding programs.

Material and methods

Plant material

Seedlings of *Eucalyptus* species, grown from certified seeds authenticated by the Brazilian Ministry of Agriculture, were got from RENASEM (National Register of Seeds and Seedlings) in 2015 (collection numbers 00605/1-6) and were kept in the municipality of São João do Triunfo, Paraná state, Brazil. Six *Eucalyptus* species were evaluated: *E. badjensis* Beuzev. & Welch, *E. benthamii* Maiden & Cambage, *E. dunnii* Maiden, *E. grandis* W.

Hill, *E. globulus* Labill and *E. saligna* Sm. Four replicates by species for each seedling were acclimatized, in an entirely randomized design, in a region with altitude of 840 m in the municipality of São Mateus do Sul (lat 25º41'00"S; long 50º17'50"W), Paraná state, Brazil. The climate in the region based on the Köppen classification is subtropical temperate (Cfb), with mean temperature from 13-21 °C (Alvares *et al.,* 2013).

For NIR spectroscopy and color studies, we collected at 15 cm from the top of trees mature leaf samples with four different ages, 6, 8, 10 and 12 months, representing approximately autumn, winter, spring and summer, respectively. On each date, four trees (repetitions) of each species were evaluated. From each tree, three leaves were analyzed for a total of 12 leaves per species. Mature leaves were collected and stored in plastic bags with moist cotton to keep them fresh for analysis.

Data acquisition was done within 24 h, while all leaves were still green. From each leaf sample, five spectra were obtained randomly from the adaxial (upper) surface, providing a total of 15 spectra from each tree and 60 spectra per species at each age. In the case of *E. saligna*, many plants died at age 8 months due to frost. Therefore, no samples were collected for analyses at this stage to avoid further damage to the existing plants.

For data acquisition, a black plate was put on the leaves to avoid external light interference. Data were collected randomly and the samples did not include the principal nervure of leaves.

Colorimetry

The colorimetric analysis was done with a CM-5 spectrophotometer (Konica Minolta, Japan) operating with standard parameters of 10° observation angle and D65 light source, with sensor aperture diameter of 3 mm. Reflectance spectra (VIS spectra) in the visible range (360-740 nm), chromatic coordinates in the green-red axis (a*) and blue-yellow axis (b*), and parameters of lightness (L*) were collected.

The data were evaluated first by computing descriptive statistics. To compare the mean colorimetric parameter values, the normal distribution of the data was checked by the Kolmogorov-Smirnov test and homogeneity of variance by the Bartlett test, both at 95% probability. After confirmation, analysis of variance was performed to verify differences between evaluated mean data, and when variances were significant, was applied the Tukey test, both with 95% probability.

Exploratory investigation was undertaken using principal component analysis (PCA) based on mean spectra of each repetition, *i.e.,* with 16 spectra for each species, to verify grouping and influence of age. In addition, the factor loading graph was evaluated to verify which wavelength had the most influence on species discrimination.

Near-infrared (NIR) spectroscopy

Infrared data were collected in a spectral range of 4000-10000 cm^{-1}, with an integrating sphere, with resolution of 4 cm^{-1}, in a FT-NIR spectrometer (Bruker Tensor 37, Bruker Optics, Ettlingen, Germany), operating in reflectance mode. Spectra were collected in wavenumber (cm^{-1}) and transformed to wavelength (nm) with the Opus 6.5 software (Bruker Optics). Data were analyzed in The Unscrambler X chemometric program (version 10.1, CAMO Software, Oslo, Norway, www.camo.com). First, principal component analysis was performed to verify possible differences between *Eucalyptus* samples, analyzing score and loading graphs. PCA was based on the NIPALS algorithm with mean centered data and cross validation. Mean spectra for each repetition (16 spectra) were analyzed in raw form and after pre-processing by derivatives of Savitzky-Golay, the first and second, both with smoothing point = 21 and polynomial order = 2.

Linear discriminant analysis (LDA)

To verify the possibility of discriminating *Eucalyptus* species in function of colorimetric parameters and VIS/NIR data, linear discriminant analysis (LDA) was tested with visible reflectance spectra, L*a*b* parameters, NIR spectra in raw form, and first and second derivatives of Savitzky-Golay (both with polynomial order = 2, smoothing point = 21).

The LDA model was constructed with Mahalanobis distance, applying equal prior probabilities and applying scores of the first 3 PCs. In function of PCA loadings, different regions in VIS/NIR spectra were tested: in VIS spectra, total reflectance spectra (350-740 nm) and the region from 700 to 740 nm; and in NIR spectra, total spectra (1000-2500 nm) and the region from 2000 to 2500 nm.

LDA was performed using three repetitions (trees) of each age and species for model construction (75% of data; 36 spectra for each species), with one repetition for external prediction (25% of data; 12 spectra for each species). Tests were also conducted applying only data from each age, and 12 months produced the best results in PCA distribution.

Table 1. Mean values and standard deviation (in parenthesis) of CIELAB parameters of *Eucalyptus* species.

Species	L*	*a**	*b**
E. badjensis	36.8 (4.0)[bc]	-5.5 (1.0)[b]	13.3 (3.8)[c]
E. benthamii	35.6 (4.0)[b]	-5.0 (0.8)[b]	8.2 (2.4)[a]
E. dunnii	32.9 (5.6)[a]	-5.3 (1.4)[b]	10.2 (4.7)[b]
E. globulus	38.0 (7.2)[cd]	-5.6 (1.6)[b]	12.3 (4.7)[c]
E. grandis	38.6 (3.3)[d]	-5.7 (2.8)[b]	21.2 (3.8)[e]
E. saligna	40.4 (2.4)[e]	-4.6 (2.6)[a]	18.0 (3.5)[d]

Same letter in each colorimetric parameter indicates no differences between species according to the Tukey´s test ($p<0.05$).

Results

Colorimetry

According to the mean color data (Table 1) of the six *Eucalyptus* species, the lowest lightness (L*) was observed for *E. dunnii* and highest for *E. saligna*. For the chromatic coordinate a* (green-red) only *E. saligna* showed different values; all the other species presented incidence of green color. The chromatic coordinate b* (blue-yellow) was more variable among the species, except for some similarity between *E. badjensis* and *E. globulus*, showing that intensity of yellow pigment influenced the final values.

The influence of age on colorimetric parameters of each species (Fig. 1) indicated some similarity within species and within ages in different species. The parameter with most variation among all samples was chromatic coordinate b*. The influence of age differed within each species.

The mean visible spectra of leaves from the six *Eucalyptus* species showed similarities (Fig. 2A). The PCA score plot (Fig. 2B) indicated formation of two groups: i) *E. badjensis*, *E. benthamii* and *E. dunnii*; and ii) *E. saligna* and *E. grandis*. Samples from *E. globulus* were mixed with other species, and some samples from *E. badjensis* and *E. dunnii* too. Loading graphs of PCA (Fig. 2C) highlighted the influence of the region from 700 to 740 nm for species distinction. By evaluating each species separately, it was possible to verify that the influence of species was greater than that of age (Fig. 3).

When the analysis was performed with mean reflectance spectra of leaves at 12 months (Fig. 4), it was possible to discriminate species, indicating that better species distinction is possible with more mature leaves.

Near infrared spectra

Mean near infrared spectra (Fig. 5A) of leaves from six evaluated *Eucalyptus* species generally had strong similarity, with only a few baseline differences. So, the spectra were preprocessed with first (Fig. 5B) and second derivative (Fig. 5C). In all graphs, it was possible to detect the influence of water content in NIR spectra in regions near 1440 nm and 1950 nm.

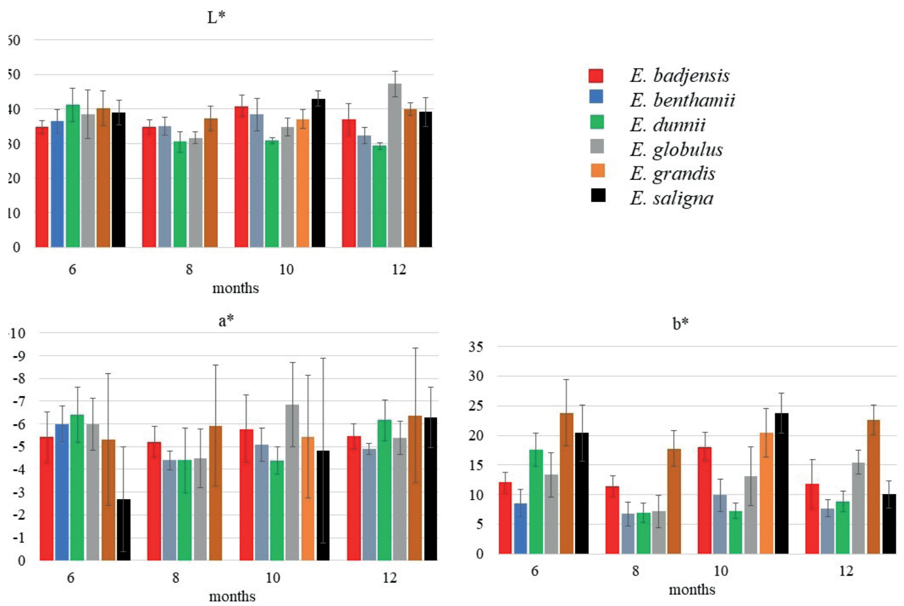

Figure 1. Mean values and standard deviations of colorimetric parameters of leaves from

Table 2. Confusion matrix and correct classification by external prediction based on the LDA model with color data and VIS/NIR spectra. For *E. saligna* n = 9, for other species n = 12.

	E. badjensis	***E. benthamii***	***E. dunnii***	***E. globulus***	***E. grandis***	***E. saligna***	**Correct classification**	***E. badjensis***	***E. benthamii***	***E. dunnii***	***E. globulus***	***E. grandis***	***E. saligna***	**Correct classification**
	L*a*b*													
E. badjensis	**2**	2	3	-	-	-	16.7 %							
E. benthamii	-	**5**	1	1	-	1	41.7 %							
E. dunnii	4	2	**2**	2	-	-	16.7 %							
E. globulus	3	2	4	**7**	-	2	58.3 %							
E. grandis	1	-	1	2	**9**	-	**75.0 %**							
E. saligna	2	1	1	-	3	**6**	66.7 %							
	VIS spectra (350-740 nm)							**VIS spectra (700-740 nm)**						
E. badjensis	**7**	1	1	1	-	-	58.3 %	**6**	-	1	-	-	1	50.0 %
E. benthamii	1	**7**	1	-	-	2	58.3 %	2	**7**	-	-	-	1	58.3 %
E. dunnii	1	-	**6**	1	-	-	50.0 %	2	1	**4**	1	-	-	33.3 %
E. globulus	3	3	3	**8**	2	1	66.7%	2	4	6	**7**	2	1	58.3 %
E. grandis	-	-	-	2	**8**	-	66.7%	-	-	-	3	**10**	1	**83.3 %**
E. saligna	-	1	1	-	2	**6**	66.7%	-	-	1	1		**5**	55.5 %
	NIR Raw spectra (1000-2500 nm)							**NIR Raw spectra (2000-2500 nm)**						
E. badjensis	**10**	-	-	2	-	1	83.3 %	**8**	1	-	-	-	-	66.7 %
E. benthamii	-	**2**	2	-	-	2	16.7 %	-	**7**	2	1	-	1	58.3 %
E. dunnii	1	5	**5**	-	2	-	41.7 %	-	3	**5**	-	1	1	41.7 %
E. globulus	1	-	3	**9**	-	-	**75.0 %**	4	-	1	**6**	-	-	50.0 %
E. grandis	-	-	1	-	**7**	1	58.3 %	-	-	-	-	**10**	-	**83.3 %**
E. saligna	-	5	1	1	3	**5**	55.5 %	-	1	4	5	1	**7**	**77.8 %**
	NIR First derivative (1000-2500 nm)							**NIR First derivative (2000-2500 nm)**						
E. badjensis	**5**	1	-	3	-	-	41.7 %	**8**	-	-	-	-	-	66.7 %
E. benthamii	-	**9**	3	-	-	2	**75.0 %**	-	**7**	6	1	-	3	58.3 %
E. dunnii	2	1	**9**	-	1	-	**75.0 %**	2	2	**3**	-	-	-	25.0 %
E. globulus	5	-	-	**8**	-	-	66.7 %	2	1	1	**10**	-	-	**83.3 %**
E. grandis	-	-	-	-	**8**	2	66.7 %	-	-	-	-	**7**	2	58.3 %
E. saligna	-	1	-	1	3	**5**	55.5 %	-	2	2	1	3	**4**	44.4 %
	NIR Second derivative (1000-2500 nm)							**NIR Second derivative (2000-2500 nm)**						
E. badjensis	**10**	1	-	3	-	-	**83.3 %**	**8**	-	3	1	-	-	66.7 %
E. benthamii	-	**5**	3	1	-	5	41.7 %	-	**5**	-	-	-	-	41.7 %
E. dunnii	-	1	**3**	-	1	-	25.0 %	2	7	**7**	-	-	3	58.3 %
E. globulus	2	-	3	**8**	-	-	66.7 %	2	-	1	**10**	-	-	**83.3 %**
E. grandis	-	-	-	-	**11**	1	**91.7 %**	-	-	-	-	**8**	3	66.7 %
E. saligna	-	5	3	-	-	**3**	33.3 %	-	-	1	1	4	**3**	33.3 %

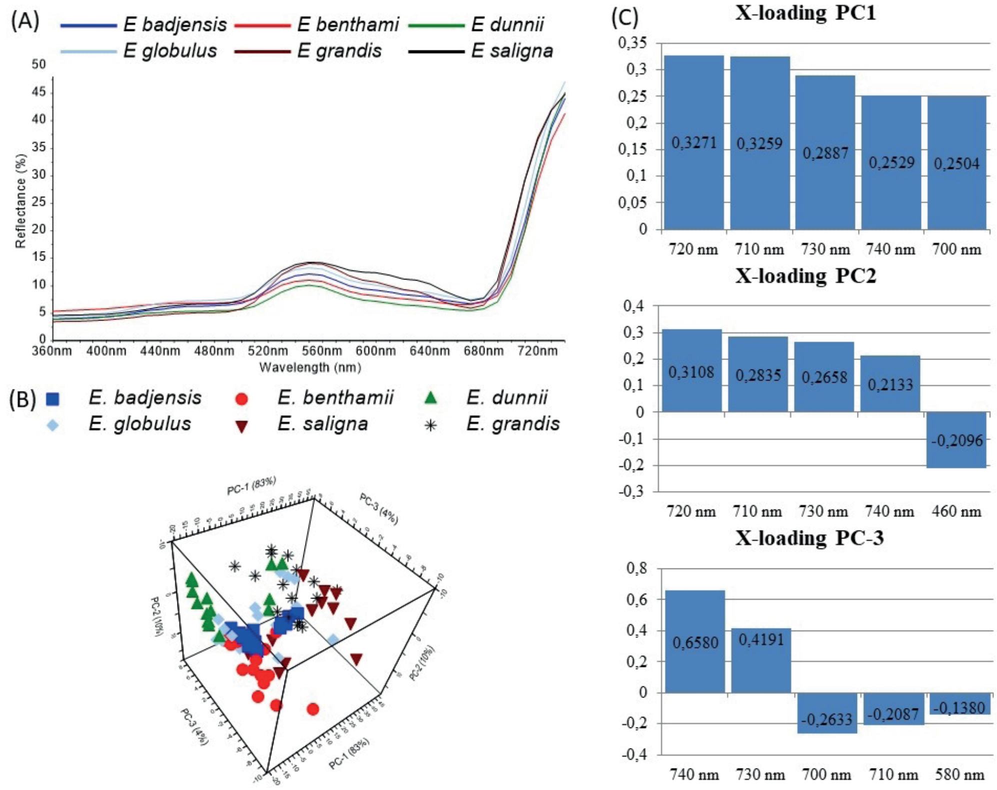

Figure 2. Mean visible reflectance spectra (A) from leaves of six *Eucalyptus* species. PCA score plot (B) and X-loading (C) of mean visible reflectance spectra of leaves of *Eucalyptus* species at

PCA score plot results (Fig. 6 A, C, E) showed differences in individual *Eucalyptus* species grouping. Loading graphs of PCA (Fig. 6 B, D, F) highlighted the influence of the region from 2000-2500 nm for species distinction. Score plot results (Fig. 7) of individual *Eucalyptus* species showed variable behavior in function of age, enabling segregation of leaves by age.

An analysis of 12-month-old samples with raw spectra (Fig. 8) indicated more similarity between *E. benthamii*, *E. dunnii* and *E. saligna* and also proximity with *E. grandis*. The most distant species were *E. globulus* and *E. badjensis*.

Linear discriminant analysis

The use of reflectance spectra or colorimetric parameters to identify species (Table 2) indicated better results for *E. grandis* (75%, L*a*b) and *E. grandis* (83.3%, Vis spectra 700-740 nm). LDA based on NIR spectra (Table 2) presented different results in function of species and pretreatment by derivatives. With raw spectra, *E. badjensis* had 83.3% correct classification in region 1000-2500 nm, and *E. grandis* in bands from 2000 to 2500 nm. After first derivative treatment, *E. globulus* had higher correct classification in the region from 2000 to 2500 nm. In turn, after second derivative treatment, *E. grandis* had the best classification of all data, with 91.7% of samples adequately discriminated.

When the LDA was performed only with data collected from leaves with 12 months of age (Table 3), colorimetric parameters and VIS spectra adequately discriminated *E. dunnii*, *E. globulus* and *E. grandis*. For the other species, the correct classification increased when the analysis was carried out in bands from 700-740 nm. Classification in function of NIR spectra presented better results with second derivative data treatment. Some confusion was found between *E. benthamii* and *E. saligna*.

Discussion

Colorimetry

Negative values of a* are related to green color and positive values of b* are associated with yellow color (Table 1). Migacz *et al.* (2018) reported that leaves of *E. globulus* are light green with some white points. According to Féret *et al.* (2011), the thickness of leaves ranges from chartaceous to coriaceous, which can influence the reflection of visible light, and the colorimetric parameters a* and b* are mainly associated with content of chlorophyll or another chemical constituent.

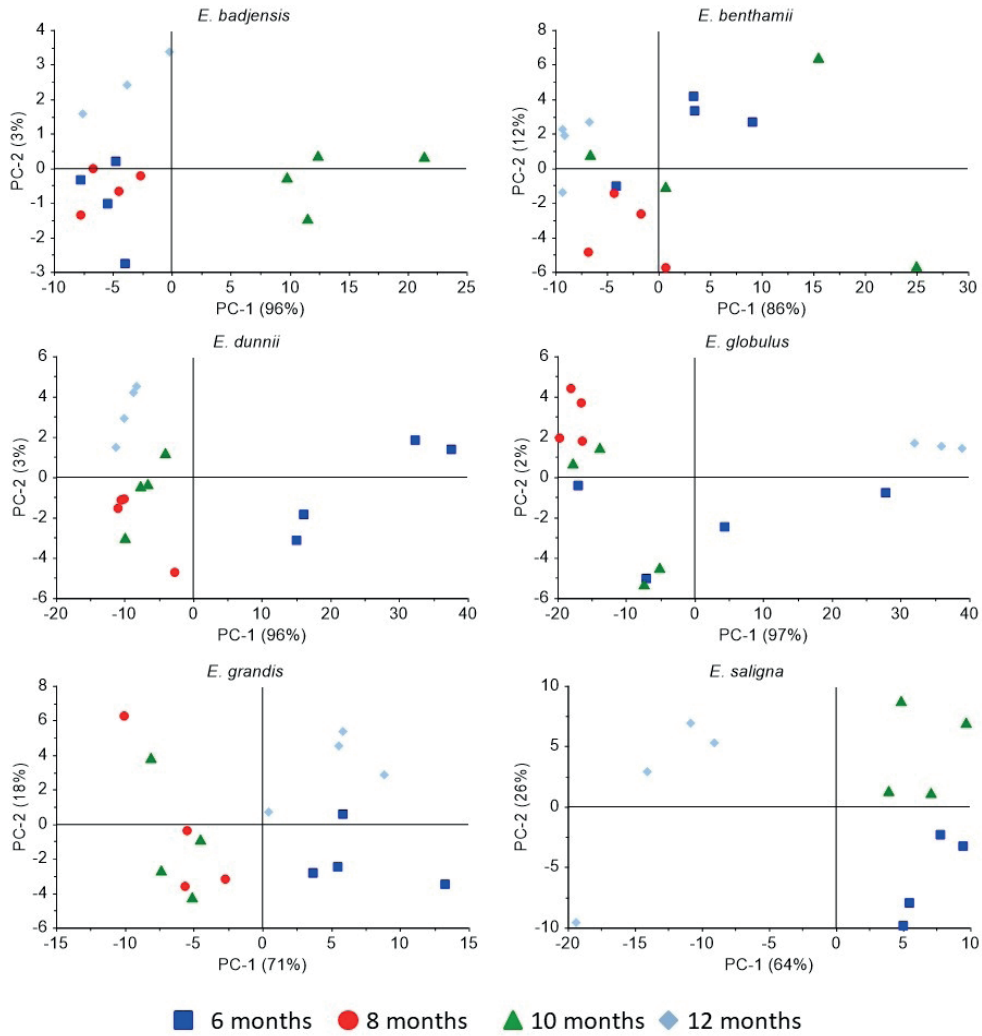

Figure 3. PCA score plot of mean visible reflectance spectra of leaves of *Eucalyptus* species at different ages.

Similar trends were observed in analysis of nine species of *Pinus* (Nisgoski *et al.,* 2015) in southern Brazil, in which lightness was different among seven species, and intensity of chromatic coordinates varied as a function of species.

Color parameters varied between species in function of age (Fig. 1). For lightness (L*), only *E. saligna* showed no influence of age. For *E. badjensis*, two groups were observed, with ages 6 and 8 and 10 and 12 months; for *E. benthamii*, leaves from plants with age 12 months had lower values, and no linear trend was observed for ages 6 and 10; for *E. dunnii*, higher values were observed at 6 months; for *E. globulus*, lightness varied among all ages; for *E. grandis*, two groups were observed, of ages 8 and 10 and 6 and 12 months.

Regarding chromatic coordinate a*, age had no influence only in *E. badjensis*. For *E. benthamii* and *E. grandis*, age 6 was different from the others; for *E. dunnii*, two groups were formed, ages 6 and 12 and 8 and 10; for *E. globulus* some similarity was observed among ages 6, 10, 12 and 8 and 12; and for *E. saligna*, all ages presented differences in green pigment.

In the case of chromatic coordinate b*, for *E. badjensis* and *E. benthamii* age 10 presented the highest values, which differed from the other ages; for *E. dunnii*, 6-month-old leaves showed intense presence of yellow pigment; for *E. globulus*, ages 6 and 10 were similar; for *E. grandis*, ages 6 and 12 had similar and higher values; and for *E. saligna*, all ages had an influence on the parameter.

As reported by previous studies, confusion among leaves from *E. benthamii* and *E. globulus* can occur, as can the leaves of *E. grandis* and *E. dunnii* (Flores *et al.,* 2016, Migacz *et al.,* 2018). However, no such confusion was observed for the colorimetric parameters in the present study.

Visual analysis of mean VIS spectra of *Eucalyptus* samples (Fig. 2A) showed similarities and peaks at green wavelengths (530-590 nm) and red wavelengths (640-670 nm). Also, a higher reflectance spectrum for *E. grandis* and *E. saligna* in the region from

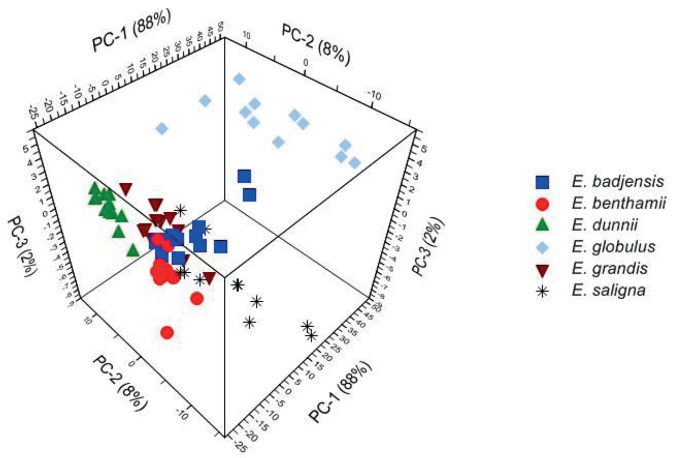

Figure 4. PCA score plot of mean visible reflectance spectra of

560-740 nm was detected. The PCA score plot (Fig. 2B) indicated a tendency for separation of all species. The variation between species is represented principally by first component (83%), and three components explained 97% of the difference. Samples from *E. badjensis*, *E. benthamii* and *E. dunnii* were more similar, which can be explained by the leaves' morphological characteristics. For example, regarding consistency, *Eucalyptus badjensis* and *E. benthamii* leaves are classified as chartaceous, whereas *E. globulus* and *E. saligna* the leaves are coriaceous. Also, sclerenchymatous fiber caps were observed in the phloem on both the adaxial and abaxial sides of *E. badjensis*, *E. benthamii* and *E. dunnii* leaves, but were absent on the leaves of other species. Finally, for *E. benthamii*, the cuticular surface is smooth while for the other species it is slightly striated (Migacz *et al.,* 2018).

The PCA factor loading graphs (Fig. 2C) identified the wavelengths with most influence in species discrimination as being the 700-740 nm region, attributed mainly to plant stress and chlorophyll concentration (Peñuelas & Filella, 1998; Carter & Knapp, 2001).

The influence of age on visible reflectance spectra of leaves from the six *Eucalyptus* species was variable (Fig. 3). For *E. badjensis*, leaves aged 10 months were distant from those of other ages; for *E. benthamii*, 10-month old leaves were distributed in all quadrants; for *E. dunnii*, it was possible to observe distinction of all ages, with proximity of 8 and 10 months; for *E. globulus*, leaves with 8 and 12 months old showed better distinction; for *E. grandis*, samples with 6 and 12 months could be individually identified; and for *E. saligna*, all samples were distinct by age.

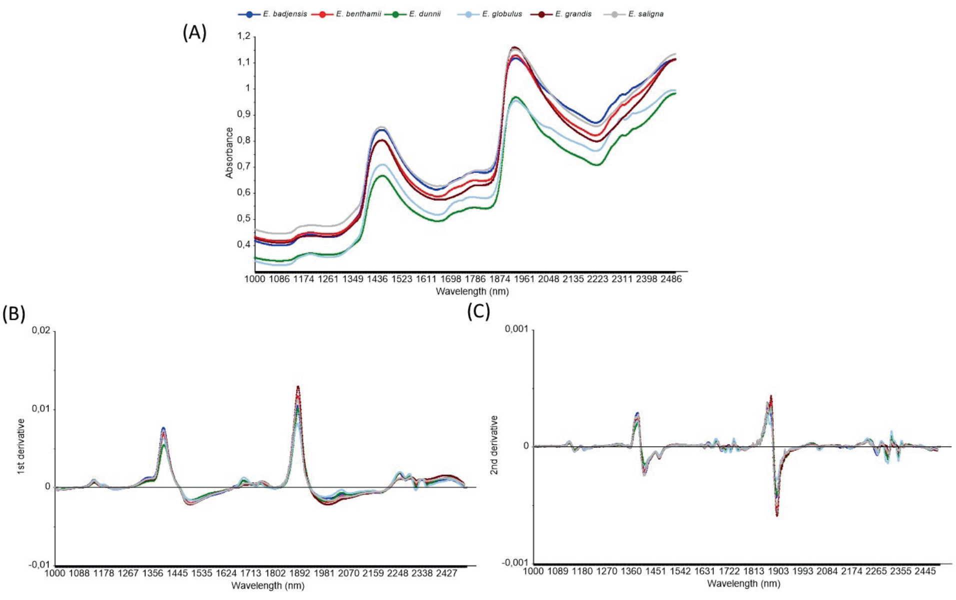

Figure 5. Mean NIR spectra of *Eucalyptus* species. Raw spectra (A), first derivative (B) and

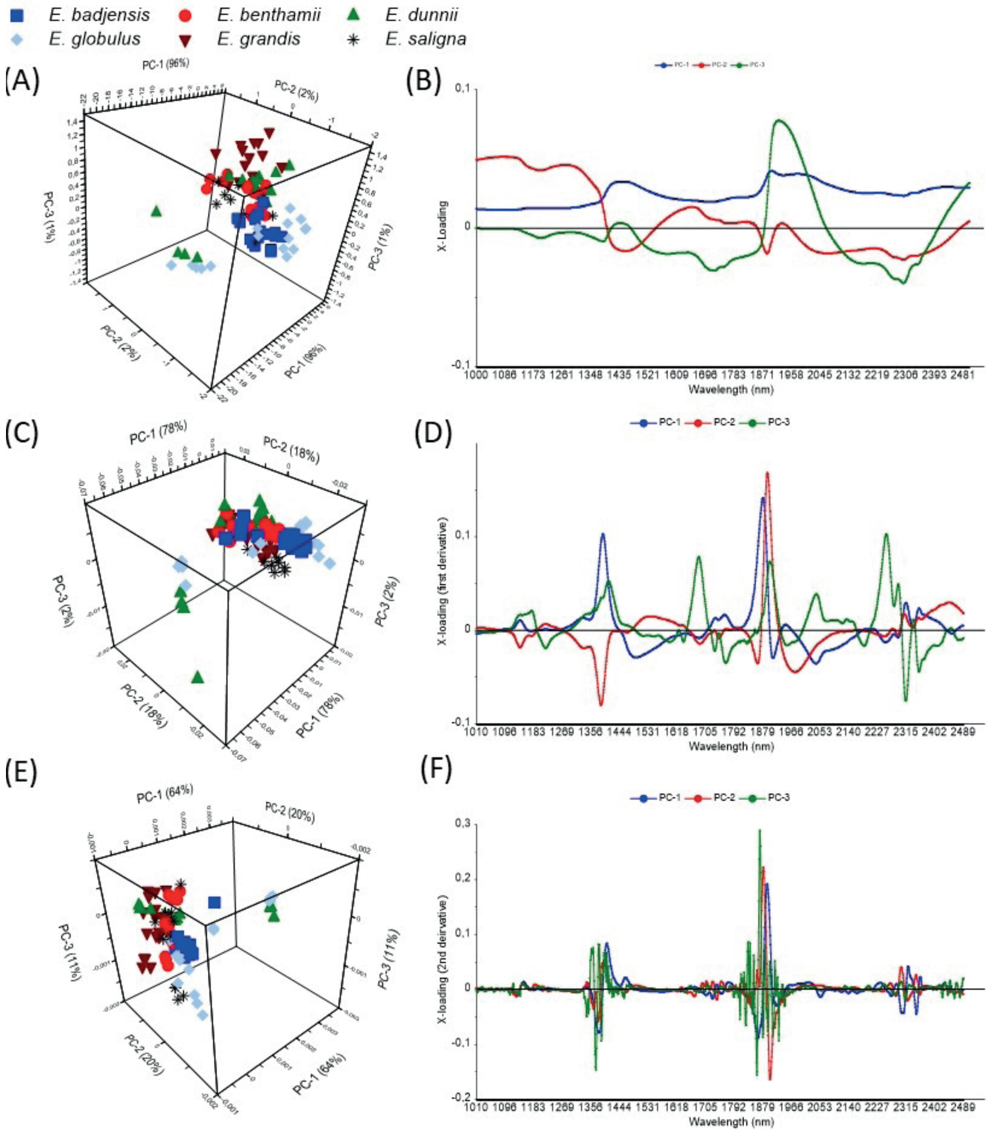

Figure 6. PCA score plot (A, C, E) and X-loading (B, D, F) of mean NIR spectra of leaves of *Eucalyptus* species at different ages. Raw spectra (A, B), first derivative (C, D) and second derivative (E, F).

Near infrared spectra

The plot of mean NIR spectra represents its similarity (Fig. 5) and reflected the influence of water content, as expected, because leaves were evaluated when green. The region from 1300-2500 nm was attributed to water and protein absorptions as well other biochemical components. Chemical bonds with water in leaves are typically present at approximately 1440 and 1950 nm (Richardson *et al.,* 2003; Homolovà *et al.,* 2013). In two other studies, wavelengths near 1360 nm and 1395 nm were associated with CH combinations; a peak at 1408 nm could be related to OH or some oil content; the band at 1463 nm was attributed to NH; the region near 1860 nm was associated with C-C bonds; wavelengths at 1881, 1894 and 2059 nm were related to cellulose; bands near 2300 nm were associated to CH from proteins and at 2323 nm to CH or CH2 starch combinations; and bands near 2347 nm were attributed to cellulose (Schenk *et al.,* 2008; Schwanninger *et al.,* 2011).

The spectra were evaluated as raw data, and then with first and second derivative treatment to reduce the baseline influence and show bands with better difference between spectra for species distinction (Fig. 5). Second derivative spectroscopy was also applied in studies of some Japanese species (Shimbori & Kurata, 2017) and coffee leaves with different ages (Mees *et al.,* 2018), with adequate results.

The PCA score and loading plot results of all samples (Fig. 6) indicated the influence of wavenumbers 2000-2500 for species differentiation and the formation of groups according to second derivative data. Score plot results (Fig. 7) of individual *Eucalyptus* species indicated that in the majority of species, leaves collected at 10

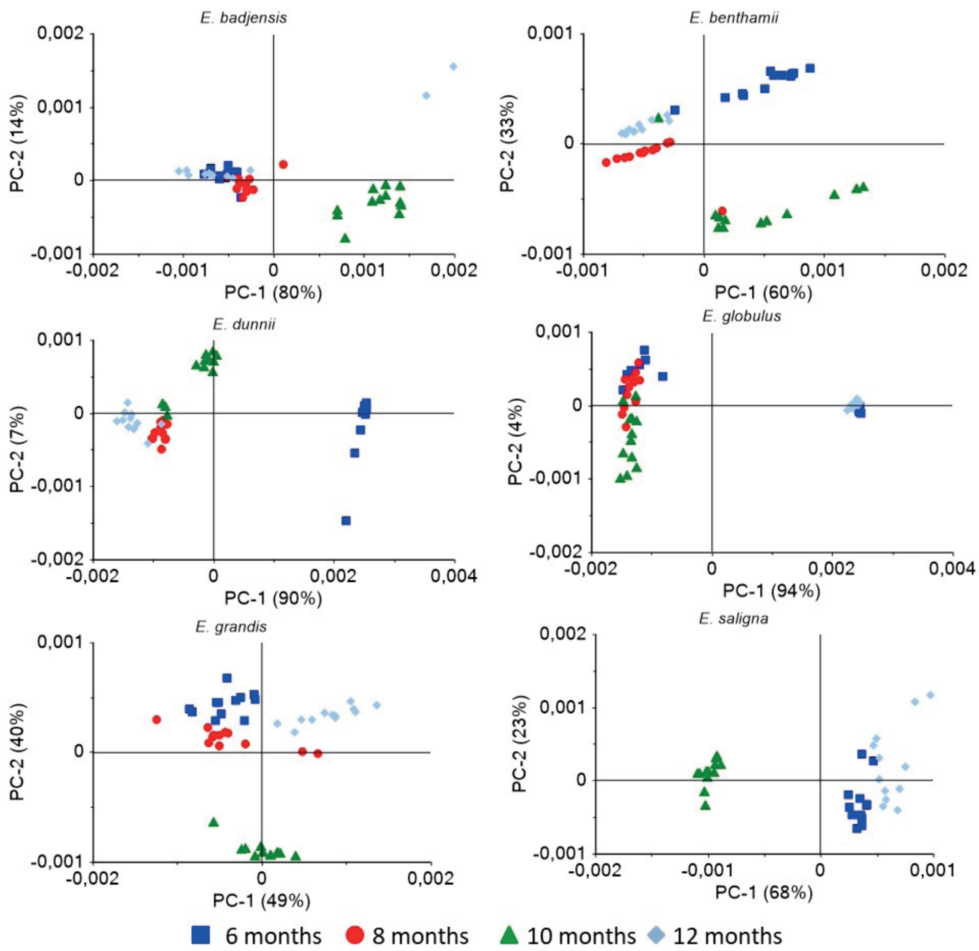

Figure 7. PCA score plot with second derivative NIR spectra of each *Eucalyptus* species at

months were distinct from the others. The collection of leaves at that age occurred in September, in the spring, when water/nutrient transport and other physiological activities of trees increase, resulting in fast growth, probably related to higher contents of chlorophyll and nitrogen along with other chemical compounds that are correlated with infrared spectra (Lohr *et al.,* 2017; Tang *et al.,* 2018), explaining the good separation.

Spectral regions are attributed to leaf characteristics, such as thickness, density, internal structure, and water and chemical content, all of which can vary due to internal metabolism and climatic characteristics of forests (Serbin *et al.,* 2012). Furthermore, it is well known that essential oil production is influenced not only by genetic inheritance, but also by factors such as environmental conditions and plant phenological stage, which can

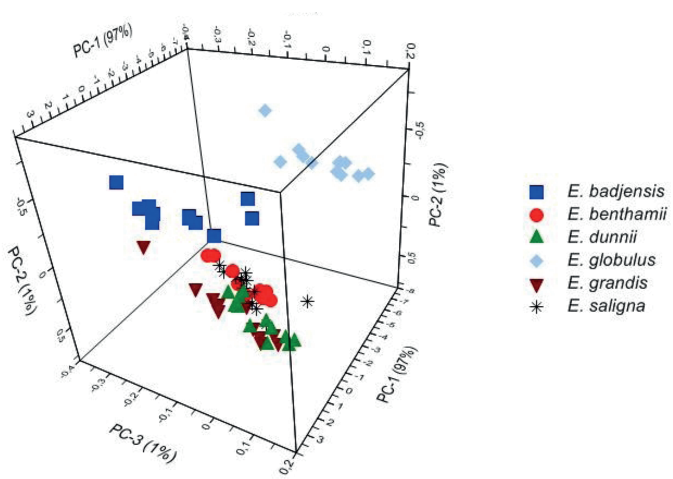

Figure 8. PCA score plot of mean NIR spectra of leaves of *Eu-*

Table 3. Confusion matrix and correct classification of external prediction based on the LDA model with color data and VIS/NIR spectra at 12 months.

	E. badjensis	***E. benthamii***	***E. dunnii***	***E. globulus***	***E. grandis***	***E. saligna***	**Correct classification**	***E. badjensis***	***E. benthamii***	***E. dunnii***	***E. globulus***	***E. grandis***	***E. saligna***	**Correct classification**
L*a*b*														
E. badjensis	**1**	1	-	-	-	1	33.3 %							
E. benthamii	-	**-**	-	-	-	-	0 %							
E. dunnii	-	1	**3**	-	-	-	**100 %**							
E. globulus	-	-	-	**3**	-	-	**100 %**							
E. grandis	-	-	-	-	**3**	-	**100 %**							
E. saligna	2	1	-	-	-	**2**	66.7 %							
	VIS spectra (350-740 nm)							**VIS spectra (700-740 nm)**						
E. badjensis	**1**	-	-	-	-	2	33.3 %	**2**	-	-	-	-	1	66.7 %
E. benthamii	-	**2**	-	-	-	-	66.7 %	-	**2**	-	-	-	-	66.7 %
E. dunnii	-	-	**3**	-	-	-	**100 %**	-	-	**3**	-	-	-	**100 %**
E. globulus	-	-	-	**3**	-	-	**100 %**	-	-	-	**3**	-	-	**100 %**
E. grandis	-	-	-	-	**3**	-	**100 %**	-	-	-	-	**3**	-	**100 %**
E. saligna	2	1	-	-	-	**1**	33.3 %	1	1	-	-	-	**2**	66.7 %
	NIR Raw spectra (1000-2500 nm)							**NIR Raw spectra (2000-2500 nm)**						
E. badjensis	**3**	-	-	-	-	-	**100 %**	**3**	-	-	-	-	-	**100 %**
E. benthamii	-	**-**	-	-	-	1	0 %	-	**-**	-	-	-	1	0 %
E. dunnii	-	-	**2**	-	-	-	66.7 %	-	-	**2**	-	-	-	66.7 %
E. globulus	-	-	-	**3**	-	-	**100 %**	-	-	-	**3**	-	-	**100 %**
E. grandis	-	-	-	-	**2**	-	66.7 %	-	-	-	-	**2**	-	66.7 %
E. saligna	-	3	1	-	1	**2**	66.7 %	-	3	1	-	1	**2**	66.7 %
	NIR First derivative (1000-2500 nm)							**NIR First derivative (2000-2500 nm)**						
E. badjensis	**3**	-	-	-	-	-	**100 %**	**3**	-	-	-	-	-	**100 %**
E. benthamii	-	**2**	-	-	-	2	66.7 %	-	**2**	-	-	-	2	66.7 %
E. dunnii	-	-	**3**	-	-	-	**100 %**	-	-	**3**	-	-	-	**100 %**
E. globulus	-	-	-	**3**	-	-	**100 %**	-	-	-	**3**	-	-	**100 %**
E. grandis	-	-	-	-	**1**	-	33.3 %	-	-	-	-	**1**	-	33.3 %
E. saligna	-	1	-	-	2	**1**	33.3 %	-	1	-	-	2	**1**	33.3 %
	NIR Second derivative (1000-2500 nm)							**NIR Second derivative (2000-2500 nm)**						
E. badjensis	**3**	-	-	-	-	-	**100 %**	**3**	-	-	-	-	-	**100 %**
E. benthamii	-	**1**	-	-	-	-	33.3 %	-	**2**	-	-	-	-	66.7 %
E. dunnii	-	-	**3**	-	-	-	**100 %**	-	-	**3**	-	-	-	**100 %**
E. globulus	-	-	-	**3**	-	-	**100 %**	-	-	-	**3**	-	-	**100 %**
E. grandis	-	-	-	-	**3**	-	**100 %**	-	-	-	-	**3**	-	**100 %**
E. saligna	-	2	-	-	-	**3**	**100 %**	-	1	-	-	-	**3**	**100 %**

modify the essential oil profile (Herraiz-Peñalver *et al.,* 2015). Differences in essential oil from natural and cultivated plants (Judzentiene *et al.,* 2015) are often linked to seasonality (Lorens-Molina & Vacas, 2015) and geographical location (Saulle *et al.,* 2018).

Lang *et al.* (2015) reported that young plants' leaves can have large chemical and structural variations, affecting in their reflectance properties, so they can be more efficient in discriminating species than leaves of old plants. Meder *et al.* (2014) observed changes in pine hybrids; Hein &

Chaix (2014), evaluating clone material from *E. grandis* × *E. urophylla,* found that inheritance of reflectance values at some wavelengths was greater than 0.5, indicating that changes in some regions are first controlled by genetics and later by chemical composition of the wood.

Evaluating the potential of NIR for discrimination of leaves of *Syzygium* species, Mendes (2014) observed grouping in function of developmental stages, but also the individual influence of each plant. In the same study, the potential of NIR for identification of nine *Eucalyptus* species and seven hybrids based on dried and ground mature leaves was described. Evaluating seedlings of eucalypts, Humphreys *et al.* (2008) concluded that NIR from fresh leaves can be applied as an alternative method in relation to chemical analysis for hybrid identification; Abasolo *et al.* (2013), testing the potential of NIR for the discrimination of *Corymbia* hybrids and their parental taxa, concluded that fresh and 8-month old leaves had the best model fit.

Grouping of *Eucalyptus* species can be based on chemical composition of leaves or genetic variations. The six species studied here are from two different sections: *E. grandis* and *E. saligna* are from the Latoangulatae section; and *E. globulus*, *E. dunnii*, *E. benthamii*, and *E. badjensis* are from the Maidenaria section (Steane *et al.,* 2011; Bayly *et al.,* 2013). Differences in morphological characteristics of leaves and epicuticular waxes along with distribution of phenolic compounds and starch are described as characteristics for the identification of these nine species (Migacz *et al.,* 2018), along with spectra. Also, genetic variation of chemical components of *E. globulus* wood from two geographic areas was found to be an adaptive response to biotic and abiotic factors (Stackpole *et al.,* 2011).

Linear discriminant analysis

When evaluating L*a*b* parameters and VIS spectra (Table 2) with LDA, better results were obtained for *E. grandis* and confusion was found among *E. globulus*, *E. dunnii*, *E. badjensis* and *E. benthamii*. The use of the region from 700-740 nm to classify species only was effective for *E. grandis*. For the other species, all spectra (350-740 nm) had better results, indicating that more data are necessary for practical application.

When the analysis was in function of NIR spectra (Table 2), the influence of species, pretreatment and spectral regions was verified. With raw spectra, better correct classification results were found for *E. badjensis* and *E. globulus* in the 1000-2500 nm region, while for *E. grandis* and *E. saligna*, the best results were achieved by the bands from 2000-2500 nm. After first derivative treatment, *E. benthamii* and *E. dunnii* with all spectra and *E. globulus* in the region from 2000-2500 nm achieved the best results. After second derivative treatment, *E. badjensis* and *E. grandis* with all bands and *E. globulus* in the region 2000-2500 nm produced the best results. *E. grandis* had the best classification based on all data, with 91.7% of samples adequately discriminated.

The performance of LDA only with data collected from leaves with 12 months of age (Table 3) increased the correct classification. Colorimetric parameters and VIS spectra adequately discriminated *E. dunnii*, *E. globulus* and *E. grandis*, but L*a*b* was not suitable for *E. benthamii*. The use of the region from 700-740 nm was most appropriate for differentiation of *E. badjensis*, *E. benthamii* and *E. saligna*.

Classification of species based on NIR spectra from leaves with 12 month of age (Table 3) was influenced by derivatives. With raw data, *E. badjensis* and *E. globulus* were adequately identified; with first derivative treatment, both species and *E. dunnii* were distinguished; and with second derivative data, only *E. benthamii* had some confusion with *E. saligna*. The other species were correctly classified.

Conclusion

The influence of age differed within each species. For colorimetric data, the parameter with most variation between all samples was chromatic coordinate b*. For reflectance spectra (VIS), leaves with age of 12 months provided the best discrimination of species. Second derivative NIR spectra produced better results of external prediction of LDA models using 12-month-old leaves.

The application of colorimetric parameters and VIS/NIR spectra for the discrimination of *Eucalyptus* species based on leaves of different ages is possible but must be refined or combined with other techniques. For practical applications, the database for comparison must be expanded. Another possibility is to dry, grind and classify leaf materials prior to spectral analysis, thus eliminating the influence of surface characteristics that can interfere in absorption/reflection.

These techniques can be applied using leaves from trees with age adequate to harvest for pulp and paper as well as with leaves suitable for extracting essential oils, increasing the aggregate value of raw materials in relation to their application for energy generation, and/or reducing the need to discard waste.

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