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ORIGINAL ARTICLE

Morphological and physiological changes during leaf ontogeny in genotypes of *Eucalyptus* young plants

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Abstract

Key message **This is a descriptive study on leaf ontogeny, showing the main morphological and physiological changes during development of leaves from young plants in three *Eucalyptus* genotypes.**

Abstract A descriptive study on key morphological and physiological features during leaf ontogeny in three genotypes—AEC 144, CO 1407 and VCC 865—of *Eucalyptus* young plants was performed. The work was developed under partially controlled environment, at two stages. First, daily photographing was used aiming at chronologically documenting noticeable changes in leaf ontogeny, such as size, shape and color, to define the reference stages of complete leaf development. Then, a time-varied split plot experiment was carried out to evaluate morphological and physiological changes in genotypes × leaf development stages relationship. The time required for complete leaf formation in *Eucalyptus* young plants varied at 44–49 days. Throughout this period, changes in leaf size, shape and color allowed us to establish four development stages, hereinafter referred to as A, B, C and D. Morphological features and color looks were described in detail at each leaf development stage. Physiological features, such as dry mass, leaf area, photosynthetic pigment content and photosynthesis rates, were increased throughout leaf development. At the early development stages, the mean values of these features were similar, by comparing the genotypes, but from stage C, however, they became larger in AEC 144 genotype than in CO 1407, which in turn were larger than in VCC 865. Decrease in sucrose hydrolysis by invertases and increase in reducing and soluble sugar content were also found during leaf ontogeny. These biochemical and metabolic changes can be interpreted as evidences of sink-to-source leaf transition, which was consolidated from stage C.

Keywords Woody plants · *Eucalyptus* spp. · Leaf development · Leaf plasticity · Sink-to-source transition

Introduction

The growing worldwide demand for *Eucalyptus* wood is one of the main causes of the planting expansion of this species in regions with different edaphoclimatic conditions. For this reason, researches have been carried out aiming to select more adaptable genotypes to edaphoclimatic conditions of each region.

A number of changes occur during plant ontogeny, in which the leaf is the organ of the plant with the greatest plasticity (Basheer-Salimia et al. 2004). In particular, morphological and physiological changes are determined by genotypic expressions, which modulate leaf plasticity, allowing the plant growth and reproduction and its adaptation to environmental changes (Reusch et al. 2005). In this sense, differences in leaf characteristics are important because leaves, as a primary carbon source for plants (Lambers et al. 2008), play an important role in adapting to environmental stress (Bréda et al. 2006).

During leaf development, morphological changes may affect light radiation interception, as well as water loss from transpiration. Physiological changes, in turn, are more related to light absorption and assimilation, as well as to

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transition from sink to source, concomitant to metabolic changes in photoassimilates degradation and synthesis. In this context, the leaf phenotypic plasticity should be seen as an integrated function of growth, morphology and physiology (Arntz and Delph 2001).

The timing of leaf development may itself be plastic (Sultan 2000) and many phenotypic responses to environmental stress factors may result from reduced growth under limited-resource conditions (Gratani and Crescente 1997). Leaf morphological and physiological properties and attributes of most plants are affected by soil moisture (Sun et al. 1996; Huang et al. 2009), salinity (Zhang et al. 2014), air temperature (Panek and Waring 1995), shade (Huang et al. 2009; Yang et al. 2014) and altitudinal gradients (Qiang et al. 2003; Li et al. 2006).

To better comprise the variations in phenotypic leaf responses to environmental stress factors, however, it is necessary to compare them with the morphological and physiological changes that occur naturally during leaf ontogeny in plants not subject to any limited-resource conditions. In addition, for trees, due to their longevity, differences among genotypes are especially important in terms of coping with environmental change (Possen et al. 2014).

In most species within the genus *Eucalyptus*, the leaves are heteroblastic, whose forms exhibit significant differences during the development stages, either in seedlings, or in young or adult plants (James and Bell 2001). Although morphological traits are described in other studies, they have not been related to the gradual and successive physiological changes that occur during leaf ontogeny (Farias et al. 2009).

Increases in chlorophyll content and photosynthesis rate have been referred as typical physiological changes that occur in leaf development. During leaf expansion, light green is usually the typical color, but the leaf may also be mixed with other colors, such as pink, purple or brown, depending on the type of anthocyanin predominant. Once completely expanded, the leaves then become dark green. Chlorophyll synthesis is closely related to leaf expansion, while photosynthesis rate grows concomitantly with the increase in chlorophyll content (Backer and Hardwick 1973).

Carbohydrate metabolism is also profoundly altered in leaf development. Young leaves are predominantly heterotrophic, which makes them partly dependent on imported carbohydrates from mature leaves. Full developed leaves, on the other hand, are autotrophic; they produce excess in photoassimilate and act as the plant's major sources of transport sugar (Turgeon 1989). This conversion from sink to source status involves changes in enzymatic activities associated with the sucrose degradation and synthesis, and marks a fundamental transition in the physiology of the leaf (Madore 1990).

In view of the above, this research was performed aiming at chronologically documenting some morphological and physiological changes that occur during leaf ontogeny in three genotypes of *Eucalyptus* spp. young plants.

Materials and methods

Three genotypes of *Eucalyptus* spp. young plants—AEC 144 (*Eucalyptus urophylla*), CO 1407 (hybrid *Eucalyptus urophylla* × *Eucalyptus grandis*) and VCC 865 (hybrid *Eucalyptus urophylla* × *Eucalyptus grandis*)—were selected for an experimental work under a partially controlled environment at the Southwest Bahia State University in Vitoria da Conquista, Bahia State, Brazil (14°53′08″ S 40°48′02″ W), from March to May 2017. The plants were grown in an area covered with colorless plastic, 100% transparent, only to avoid the influence of precipitation, and lined laterally by a mesh containing perforations for air circulation. During the experimental period, the mean temperature and relative humidity were 22 °C (20–24 °C) and 68% (63–73%), respectively.

First, 100-day-old seedlings were produced in small tubes (54 cm^3) and then transplanted in 15 L pots (one plant per pot) containing yellow oxysol with sandy–clay texture fertilized according to the soil chemical analysis and nutrient demand of Eucalyptus (Ribeiro et al. 1999). Optimum water supply was ensured, maintaining soil moisture close to field capacity through daily irrigation, to prevent water stress conditions for plant growth.

This study was carried out at two stages. First, ten young plants from each genotype were chosen for daily photographing, aimed at chronologically documenting noticeable changes in leaf ontogeny, from the early leaf budding up to its full expansion. Images were taken always at 9–10 a.m. by a Canon PowerShot SX520HS camera. The main changes in leaf color, shape and size were identified and then the reference stages of complete leaf development were defined.

After that, an experiment was carried out to evaluate morphological and physiological changes in genotypes × leaf development stages relationship. Treatments were arranged by a completely randomized design, using a time-varied split plot 3 × 4—three genotypes (AEC 144, CO 1407 and VCC 865) and four leaf development stages (A, B, C and D)—with three replicates, one plant per pot. The four leaf development stages were previously defined as a result of the first work stage.

Length, width, total area, dry mass and invertase activity were evaluated at all leaf development stages. For length and width measurements we used a ruler, while total leaf area was measured by a leaf area meter (LI-COR, model LI-3100). Leaf dry mass was obtained in oven set to 70 °C, for 48 h. Photosynthetic pigments were extracted from the leaves using acetone 80%; chlorophylls (*a* and *b*) and carotenoid content were determined

by spectrophotometric analysis (Arnon 1949). Total invertase (acid and neutral) activity was evaluated by an in vivo method (Cairo et al. 2009), whose protocol is based on 50 mg leaf tissue samples added to enzymatic assays, which were kept in a water bath (37 °C) for 1 h. The composition for acid invertase assays (both vacuole and cell wall enzymes) was 200 µL sodium acetate buffer 1.0 M pH 4.7, 100 µL $MgCl_2$ 0.1 M, 400 µL sucrose 1.0 M and water added up to 2000 µL. A similar composition was used for neutral invertase assays, although slightly modified with potassium phosphate buffer at pH 7.5. Reducing sugar content was determined according to Miller (1959).

Photosynthesis rate, stomatal conductance and internal CO_2 concentration were evaluated at 9–11 a.m. using an infrared gas analyzer (IRGA LI-6400, LI-COR®, Nebraska/USA) at B, C and D development stages. Preliminary measurements had proved that a natural photosynthetic photon flux density (PPFD) of at least 1000 µmol photons m^{-2} s^{-1} provided full (> 95%) photosynthesis saturation. Thus, the leaves were irradiated at about 1000 µmol m^{-2} s^{-1} provided by a Björkman lamp (Hansatech, Kings Lynn, UK). The external CO_2 concentration from the air entering the chamber was maintained at 360 µmol mol^{-1} and the light-satured photosynthesis rate on leaf area basis was measured. Leaf temperature during measurements ranged from 25 to 30 °C.

Reducing and soluble sugar content were also determined only in leaf tissues at B, C and D development stages. Sugar extracts of the dried leaf tissues were three times centrifuged (10,000×*g*) for 30 min with potassium phosphate buffer 0.1 M. The supernatant were collected and the sugar content was determined according to Miller (1959) for reducing sugar and Yemm and Willis (1954) for soluble sugar.

Data were submitted to analysis of variance for statistical analysis and means were compared by Tukey's test ($p < 0.05$).

Results and discussion

Leaf development stages description

Stage A (duration: 12–15 days)

In the initial leaf ontogeny, the leaf bud first expands in width, and then soon expands in length quickly, acquiring a shape similar to a pin, which can reach up to 1–2 cm, depending on the genotype (Fig. 1).

After a few days (3–4 days, depending on the genotype), the leaf bud growth is followed by a sudden longitudinal growth of the petiole, which starts to exhibit two parts: one proximal (the petiole itself), and the other part narrow and distal. From this distal part, a composite blade is formed, resulting from the external scales opening, which grows in length, width and thickness (Fig. 2). Thus, the leaf blade starts to be established, and the axillary bud, which was in a dormancy period, becomes easily noticeable, being able to produce new leaves later.

According to Fahn (1990), leaf blade development occurs during leaf primordia growth in length and thickness, resulting from the continuous division of the cells of the margins, forming the marginal meristem. "Blastozone" is the term that has been proposed to designate regions of the shoot competent for organogenesis. It is argued that

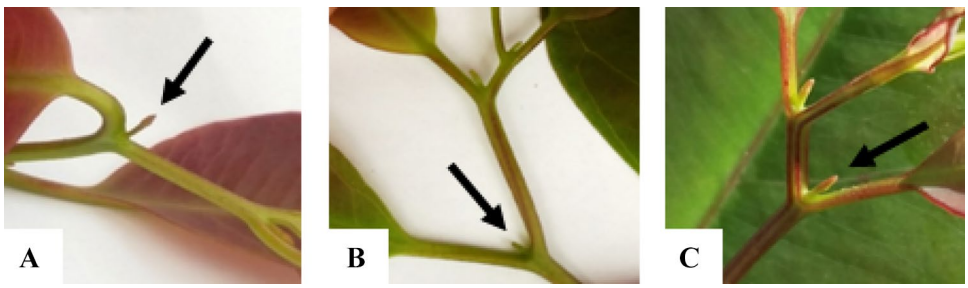

Fig. 1 Leaf bud expansion during initial leaf development (stage A) in *Eucalyptus* young plants, genotypes AEC 144 (**a**), CO 1407 (**b**) and VCC 865 (**c**)

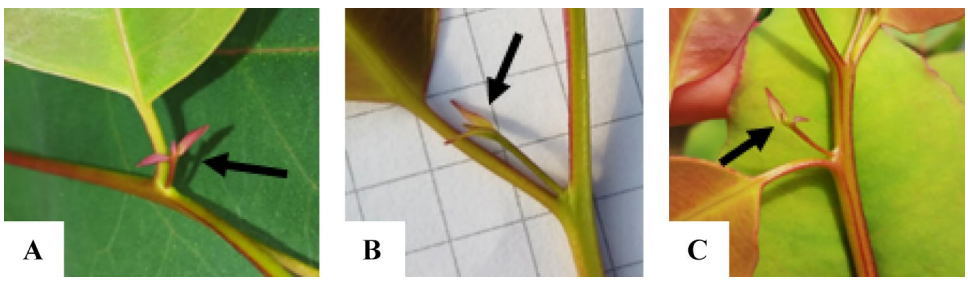

Fig. 2 Leaf blade opening during initial leaf development (stage A) in *Eucalyptus* young plants, genotypes AEC 144 (**a**), CO 1407 (**b**) and VCC 865 (**c**)

the notion of "marginal meristems" is based on the cell theory and thus may not be appropriate to elucidate the process of organ formation (Hagemann and Gleissberg 1996). The marginal growth varies among the regions of leaf primordium, so that, in petiole leaves, such as those of Eucalyptus, leaf growth is repressed at the base, from which the petiole originates. From the first marginal cell divisions, the leaf blade tissues originate.

The petiole is fixed to the base and slightly flattened and ribbed. Due to its variegated coloring, the petiole tends to exhibit two or more colors throughout its growth. In a single plant of CO 1407 genotype, petioles can be totally green, but may also exhibit reddish and brownish tones only in veins that form terminal angles, with greenish tones in the central region. In AEC 144 and VCC 865 genotypes, totally reddish and brownish petioles may also occur. In stage A, besides the leaflets opening, an extensive elongation of the petiole occurs and can reach 1.0–3.5 cm in length (Fig. 3).

While petiole elongation occurs, leaf blades also expand in width and length, forming single and entire blades, quite narrow and positioned in the vertical position, with an acute apex facing upward. The central vein becomes prominent and light green in all three genotypes, exhibiting smooth margins in AEC 144 and VCC 865, and undulating in CO 1407. In addition, genotypes AEC 144 and VCC 865 exhibit strong anthocyanin staining, which is more brownish in CO 1407. These anthocyaninic shades persist until the transition to stage B (Fig. 4).

Stage B (duration: 10 days)

At this stage, a leaf blade expansion, which is larger in length than in width, occurs forming a typical laminar structure. With alternate phyllotaxy, a leaf color changing occurs throughout the stage B, starting from initial reddish anthocyaninic tones, as in VCC 865 and AEC 144 genotypes and brownish in CO 1407, until they acquire a solid green color, when the leaf development reaches the next stage. Previous studies by Hallé et al. (1978) on leaf ontogeny in rubber tree were our reference to point out this color change in the leaves from genotypes of Eucalyptus young plants as one of the main events at stage B. According to these authors, B1 and B2 are the stages in which a notable change in the predominant color during leaf ontogeny in that species occurred, when reddish tones were gradually replaced by green tones.

At stage B, the following morphological features are observed: glabrous leaf blade, with oval and rounded shape, and smooth edges, which may exhibit soft undulations, as found in CO 1407 genotype. In all genotypes, the apex has an acute-acuminate form, while the base is unevenly obtuse or rounded. Leaf blade dimensions may vary from 4.0 to 6.0 cm in length and from 1.5 cm to 3.5 cm in width (Fig. 5).

Stage C (duration: 8–10 days)

At this stage, the leaf blade expands greatly and the veins become more proeminent. In all three genotypes, the green color becomes widely predominant, with several translucent

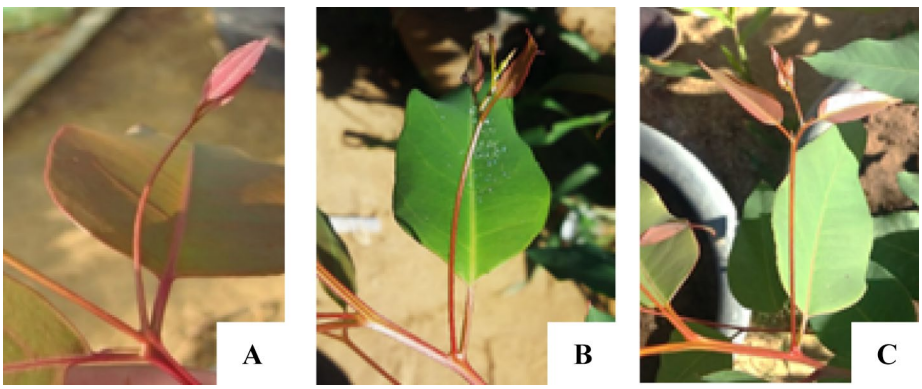

Fig. 3 Extensive elongation of the petiole at stage A, during initial leaf development in *Eucalyptus* young plants, genotypes AEC 144 (**a**), CO 1407 (**b**) and VCC 865 (**c**)

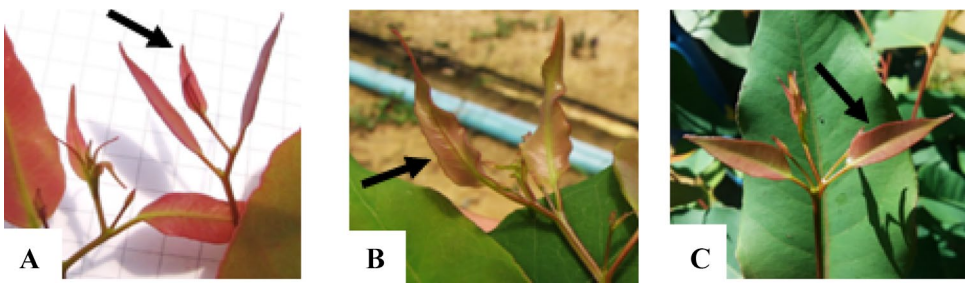

Fig. 4 Leaf blades in stage A, showing light green coloration in central veins and anthocyanin tonalities in most of the leaf surface, during initial leaf development in *Eucalyptus* young plants, genotypes AEC 144 (**a**), CO 1407 (**b**) and VCC 865 (**c**)

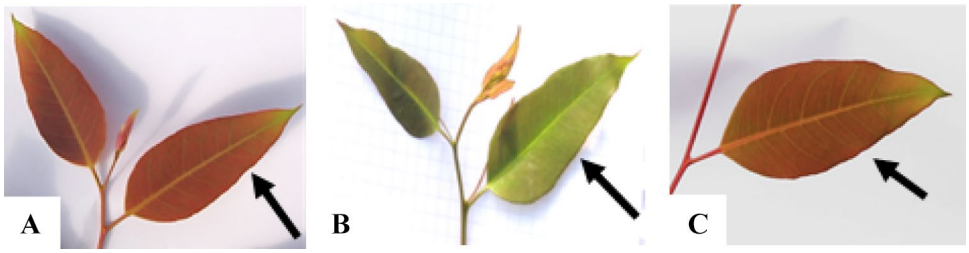

Fig. 5 Some morphological features of leaf development at stage B in *Eucalyptus* young plants, genotypes AEC 144 (**a**), CO 1407 (**b**) and VCC 865 (**c**): glabrous leaf blade, with oval and rounded shape, and smooth edges, which may exhibit soft undulations, as in CO 1407 genotype

points, which are not always easily noticeable. The appearance of these translucent points is considered as usual in leaves of plants from Myrtaceae family, and indicates the presence of secretory cavities of essential oils (Castro and Machado 2012).

The leaf margin still shows reddish tones which indicate the remaining presence of anthocyanins, in AEC 144 and VCC 865 genotypes. Nevertheless, since the leaf margin in CO 1407 no longer has reddish tones, the leaf color is given as totally green.

In all three genotypes, the leaf blade length is 5–8 cm. On the other hand, the width is 7–8 cm in AEC 144 and VCC 865, and 4–5 cm in CO 1407. In CO 1407 genotype, the leaf blade length is much larger than width; furthermore, as the width becomes even narrower toward its apex, this leaf is classified as lanceolate. In the other genotypes, nevertheless, the leaf is classified as an oval form, given that the leaf blade base is wider and the apex is not so narrow (Fig. 6).

Stage D (duration: 14 days)

When the leaves reach this last stage, they appear more consistent, in structural terms, and become fully expanded. Comparing the three genotypes, CO 1407 is one whose leaves show a longer look, reaching 12–14 cm in length and 5–7 cm in width. AEC 144 is one whose leaves are wider, reaching 12–14 cm in length and 8–10 cm in width. Finally, the VCC 865 is the one whose leaves are intermediate in relation to the other two, reaching 10–12 cm in length and 7–8 cm in width (Fig. 7).

Previous studies have shown that differences among Eucalyptus genotypes related to anatomical and morphological changes during foliar ontogeny are relatively usual (Souza 2008). There are also reports that differences among *Eucalyptus camaldulensis* genotypes during leaf ontogeny may be related to variations in plant root morphology, which may affect the ability of water and nutrient uptake, thus causing differences in leaf morphology (James 1995).

Morphophysiological changes at leaf development stages

The dry mass increased followed leaf development, as expected, reaching the largest values at leaf maturity (stage D). On average, this increase was about 90% in relation to stage A. The highest total dry mass accumulation during leaf ontogeny was found in AEC 144 genotype, although there were no differences among the genotypes at stages A and B. The leaf dry mass of AEC 144 genotype became larger than those of the other genotypes only at stages C and D, when it reached 0.35 g and 0.48 g respectively. In contrast, the lowest leaf dry mass accumulation was in the VCC 865 genotype (Table 1).

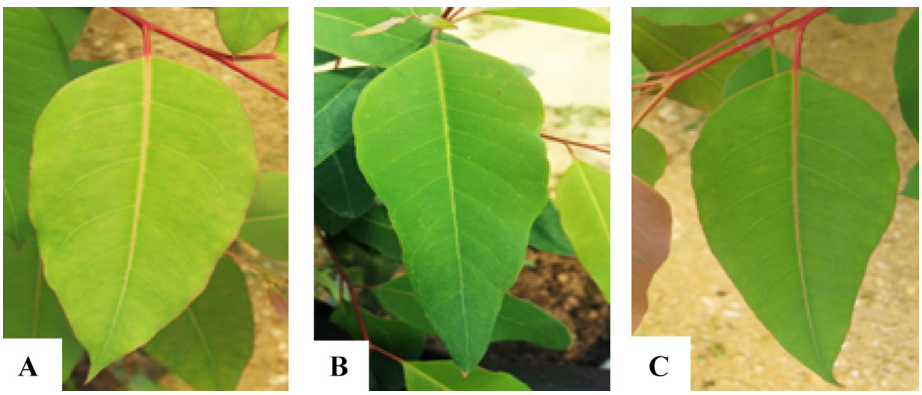

Fig. 6 Leaf development at stage C in *Eucalyptus* young plants, genotypes AEC 144 (**a**), CO 1407 (**b**) and VCC 865 (**c**)

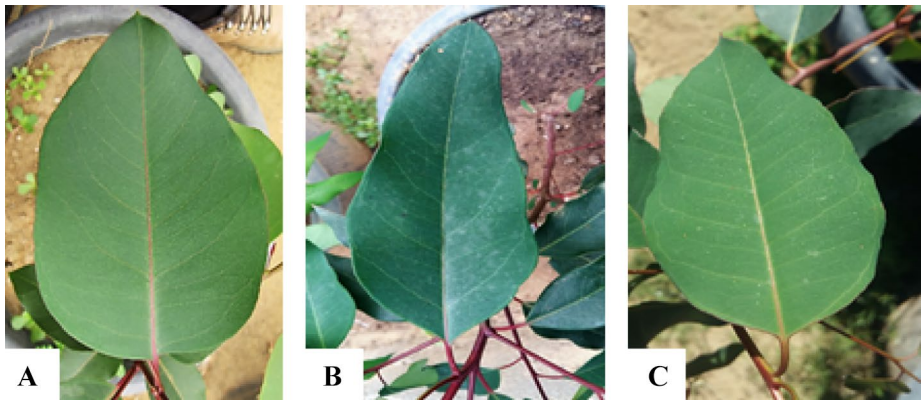

Fig. 7 Final stage of leaf development (stage D) in *Eucalyptus* young plants, genotypes AEC 144 (**a**), CO 1407 (**b**) and VCC 865 (**c**)

Table 1 Leaf dry mass at different development stages, in *Eucalyptus* young plants, genotypes AEC 144, CO 1407 and VCC 865

Genotypes	Leaf dry mass (g) at different development stages*			
	A	B	C	D
AEC 144	0.052 Ad	0.162 Ac	0.345 Ab	0.482 Aa
CO 1407	0.047 Ad	0.163 Ac	0.310 Bb	0.448 Ba
VCC 865	0.031 Ad	0.138 Ac	0.260 Cb	0.352 Ca

*Within each column, mean values followed by the same capital letter indicate that genotypes are not different. Within each line, mean values followed by different lowercase letters indicate significant variation among the leaf development stages. Mean values were compared using Tukey's test ($p < 0.05$)

Table 2 Leaf area at different development stages, in *Eucalyptus* young plants, genotypes AEC 144, CO 1407 and VCC 865

Genotypes	Leaf area (cm^2) at different development stages*			
	A	B	C	D
AEC 144	1.995 Ad	13.464 Ac	40.482 Ab	70.932 Aa
CO 1407	1.861 Ad	14.496 Ac	33.902 Bb	53.785 Ba
VCC 865	1.645 Ad	13.838 Ac	30.502 Cb	48.279 Ca

*Within each column, mean values followed by the same capital letter indicate that genotypes are not different. Within each line, mean values followed by different lowercase letters indicate significant variation among the leaf development stages. Mean values were compared using Tukey's test ($p < 0.05$)

From stage C, leaf maturation occurs more rapidly, and sink-to-source transition becomes more evident. During this transition, due to the combined effects of biochemical and structural changes on the development of photosynthetic capacity (Miguel et al. 2007; Niinemets et al. 2012), the leaf increases its ability for photoassimilate synthesis and export. The cessation of sugar import involves blockage of unloading from the major veins for sometime during the leaf development (Shakya and Lal 2018).

In general, environmental-limited conditions may cause negative effects on plant physiology and metabolism. Thus, water and nutrient availability are the factors referred to for determining phytomass accumulation and partitioning, and may influence both dry mass allocation on leaf and the time required for its complete development (Gonçalves and Passos 2000).

Referring to leaf area, there were no differences among the genotypes during the initial stages A and B. At stages C and D, however, leaf area was higher in AEC 144 genotype, followed by CO 1407, which, in turn, was larger than in VCC 865 (Table 2).

The leaf area expansion is usually considered as one of the important requirements to enlarge light harvesting. There are inherent differences among various functional types of plants, in terms of biomass allocation costs for foliage investment. Unlike herbaceous plants, where there are no woody support tissues, these costs are higher in tree species, which have a structure of more consistent and long-living expensive branch and stems (Niinemets 2010). Leaf area expansion, obviously, is closely related to some of the major plant physiological events, such as photosynthesis, transpiration and carbon flow (Cleugh et al. 2007). In addition, studies on several species, such as sunflower (Carvalho 2004), sugarcane (Almeida et al. 2008), melon (Maia et al. 2009) and maize (Araújo Júnior et al. 2010) report that leaf area expansion also depends on changes in the metabolism of sucrose synthesis and hydrolysis, which occur during sink-to-source transition (Marafon 2012).

Throughout the ontogeny, the leaves gradually became green, as a result of increase in chlorophylls *a* and *b* content, with no differences among the genotypes at stages A and B. Different performances among the genotypes were found at stage C, when the chlorophyll *a* content was higher in AEC 144 genotype, followed by CO 1407, which was higher than in VCC 865. A similar performance among the genotypes was also found in both chlorophyll *b* and carotenoid content, although it was only at stage D (Table 3).

Lower chlorophyll *a*/*b* ratio in genotype AEC 144 is a result of an increase in chlorophyll *b* content. This is particularly important when the plant is submitted to shaded

Table 3 Chlorophylls *a*, *b*, total, *a*/*b* ratio and carotenoid at different leaf development stages in *Eucalyptus* young plants, genotypes AEC 144, CO 1407 and VCC 865

Genotypes	Leaf development stages		
	B	C	D
Chlorophyll *a* (mmol m^{-2})*			
AEC 144	0.652 Ac	1.172 Ab	2.575 Aa
CO 1407	0.635 Ac	1.154 Bb	2.246 Ba
VCC 865	0.628 Ac	1.133 Cb	1.931 Ca
Chlorophyll *b* (mmol m^{-2})*			
AEC 144	0.253 Ac	0.415 Ab	0.958 Aa
CO 1407	0.244 Ac	0.403 Ab	0.810 Ba
VCC 865	0.236 Ac	0.390 Ab	0.704 Ca
Chlorophyll total (mmol m^{-2})*			
AEC 144	0.905 Ac	1.587 Ab	3.533 Aa
CO 1407	0.879 Ac	1.557 Ab	3.056 Ba
VCC 865	0.864 Ac	1.523 Ab	2.635 Ca
Chlorophyll *a*/*b* ratio			
AEC 144	2.577	2.824	2.688
CO 1407	2.602	2.864	2.773
VCC 865	2.661	2.905	2.743
Carotenoid (mmol m^{-2})*			
AEC 144	0.451 Ac	0.866 Ab	1.735 Aa
CO 1407	0.438 Ac	0.849 Ab	1.496 Ba
VCC 865	0.420 Ac	0.838 Ab	1.144 Ca

*On each pigment: within each column, mean values followed by the same capital letter indicate that genotypes are not different. Within each line, mean values followed by different lowercase letters indicate significant variation among the leaf development stages. Mean values were compared using Tukey's test ($p < 0.05$)

Table 4 Net photosynthesis, stomatal conductance and internal CO_2 concentration at different leaf development stages in *Eucalyptus* young plants, genotypes AEC 144, CO 1407 and VCC 865

Genotypes	Leaf development stages		
	B	C	D
Net photosynthesis (μmol CO_2 m^{-2} s^{-1})*			
AEC 144	6.043 Ac	13.540 Ab	17.515 Aa
CO 1407	5.887 Ac	12.783 Bb	15.791 Ba
VCC 865	5.675 Ac	12.371 Cb	14.081 Ca
Stomatal conductance (mol m^{-2} s^{-1})*			
AEC 144	0.033 Ac	0.137 Ab	0.168 Aa
CO 1407	0.029 Ac	0.120 Bb	0.151 Ba
VCC 865	0.026 Ac	0.104 Cb	0.139 Ca
Internal CO_2 concentration (μmol mol^{-1})*			
AEC 144	249.6 Ac	297.9 Ab	331.3 Aa
CO 1407	243.3 Ac	271.8 Bb	314.0 Ba
VCC 865	238.8 Ac	248.3 Cb	285.5 Ca

*For each variable, within each column, mean values followed by the same capital letter indicate that genotypes are not different. Within each line, mean values followed by different lowercase letters indicate significant variation among the leaf development stages. Mean values were compared using Tukey's test ($p < 0.05$)

environment, because higher chlorophyll *b* levels allow light interception in wider wavelength bands (Gonçalves et al. 2001). On the other hand, the higher chlorophyll *a*/*b* ratio in genotype VCC 865 leaves may be interpreted as an indication of higher ability to perform high rates of photochemistry when the plant is under full illumination, due to lower light absorbance by PSII, which reduces high radiation stress (Barros et al. 2011).

The higher carotenoid content found in genotype AEC 144 is compatible with its role in maximizing light capture in eventual shade environments. Carotenoids play a secondary role in photosynthesis, serving as accessory pigments that absorb light between 400 and 500 nm (between blue and green), transfering it to chlorophyll molecules, and have an additional function to give structural stability to the assembly of light-harvesting complexes (Lal 2018).

The leaf photosynthetic pigment content determines its ability for light harvesting. Typically, the pigment content per unit of dry mass increases with leaf development (Niinemets 2010). Larger leaves have sufficient thickness to provide better distribution of chloroplasts and more photosynthetic pigments, which may improve the photosynthetic rate, due to the increase in quantum capture potential per unit time (Conforto et al. 2011). Studies by Krause et al. (1995) with tropical tree species showed that young leaves have less than 50% chloroplastidic pigments per unit area compared to mature leaves. According to these researchers, in spite of the greater investment in photoprotective pigments, such as α-carotenes and pigments linked to the xanthophyll cycle, young leaves are more susceptible to damage in photosystem II, detected by the photoinhibition process.

Throughout leaf development, photosynthesis rate, stomatal conductance and internal CO_2 concentration became progressively higher. Although without differences among the genotypes at stage B, from stage C these variables became higher in AEC 144 genotype, followed by CO 1407, which was higher than in VCC 865 (Table 4). The photosynthesis rate increase during the leaf development stages is compatible with both dry mass and chlorophyll performances, which were also evaluated in this study. On the other hand, the increase in photosynthesis rate during leaf development, followed by increases in both stomatal conductance and internal CO_2 concentration, corroborates previous studies that report a close correlation between these variables (Farquhar and Sharkey 1982; Miyazawa and Terashima 2001; Tominaga and Kawamitsu 2015).

In young rubber tree, low photosynthesis rates found during early leaf ontogeny have been attributed to a conjunction of factors such as high respiration rate and stomatal resistance (Pita et al. 1988; Schwob et al. 1998), CO_2

compensation point (Bergonci 1981) and low chlorophyll content (Pita et al. 1988; Miguel et al. 2007). Our results allow us to agree that the low chlorophyll content during early leaf development should be the real determinant for the low photosynthesis rates in Eucalyptus genotypes.

At stage B, low photosynthesis rates suggest that at early development stages, the young leaf can be considered as a typical sink, whose sugar demand should be supplied by mature source leaves. Photosynthesis rate at these stages can therefore also be interpreted as a limiting factor for the leaf playing its role as a source, thus supplying the assimilate demand of other sink tissues.

In *Castanopsis sieboldii*, a climax species of evergreen tree with broad leaves commonly found in eastern subtropical Asia, the rate of photosynthesis was found to increase with leaf age and reached its maximum a few days before the end of full leaf expansion (Miyazawa and Terashima 2001). These researchers reported that in the evergreen broad-leaved trees, mechanical protection of mesophyll cells has priority over the efficient CO_2 transfer and quick construction of the chloroplast. According to Pimentel (1998), the photosynthetic activity is a function of the number of chloroplasts, which can be arranged both horizontally (greater leaf area) and vertically (greater leaf thickness). During leaf development, the photosynthetic activity per unit of leaf area is higher in leaves whose expansion has just been completed, and then decreases with leaf senescence (Pimentel 1998).

Total invertase showed high activity in leaf primordia and became lesserthroughout the leaf development stages. The enzyme activity decrease at stage A–B transition was more pronounced than at the following stages. Comparing the genotypes, the leaf invertase activity was higher in AEC 144 than in the other two genotypes (Fig. 8).

Sucrose hydrolysis by invertase plays an important role to supply reducing sugar demand in sink tissues (Koch 2004). The higher invertase activity at early leaf development stages is compatible with its transitory sink status, marked by poor chlorophyll content, low photosynthesis rate and sucrose import (Batta et al. 2008).

Decreased invertase activity, concomitant with increases in chlorophyll content and photosynthesis rate throughout the leaf development stages, can be interpreted as an indication of maturation process progress, once the leaf gradually becomes autotrophic and self-sufficient for its own sucrose demand. According to Pimentel (1998), dicotyledon leaves are usually referred to as autotrophics and sucrose exporters only when they reach 30–60% of their maximum leaf area—unlike monocotyledon leaves, as in sugarcane, where the sucrose import is maintained until 90% of the maximum leaf area is reached. Based on this assumption, we have considered that the leaves of the Eucalyptus genotypes in this study should have become sources at stages B–C transition, when then reached 57.8%, 63.0% and 63.2% of maximum leaf area

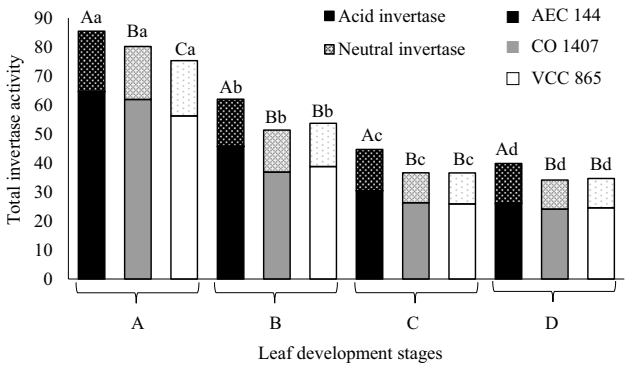

Fig. 8 Total invertase activity (acid + neutral) at different leaf development stages in *Eucalyptus* young plants, genotypes AEC 144, CO 1407 and VCC 865. Data are expressed as µmol reducing sugar g^{-1} fresh weight $hour^{-1}$. Within each leaf development stage, mean values followed by the same capital letter indicate that genotypes are not different. Within each genotype, mean values followed by different lowercase letters indicate significant variation among the leaf development stages. Mean values were compared using Tukey's test ($p < 0.05$)

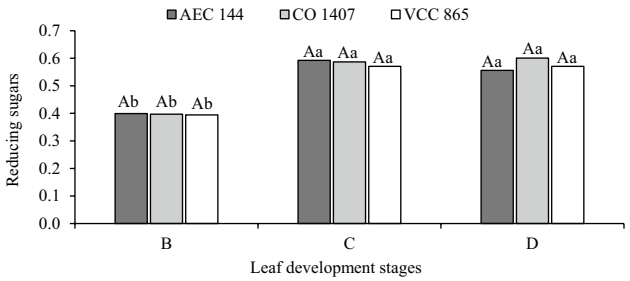

Fig. 9 Reducing sugar content at different leaf development stages in *Eucalyptus* young plants, genotypes AEC 144, CO 1407 and VCC 865. Data are expressed as µmol reducing sugar g^{-1} fresh weight. Within each leaf development stage, mean values followed by the same capital letter indicate that genotypes are not different. Within each genotype, mean values followed by different lowercase letters indicate significant variation among the leaf development stages. Mean values were compared using Tukey's test ($p < 0.05$)

in AEC 144, CO 1407 and VC 865 genotypes, respectively (Table 2).

At all leaf development stages, acid invertase activity was higher than neutral invertase (Fig. 8). This performance corroborates previous studies that point to acid invertase as a key enzyme for phloem unloading and hexose maintenance, especially in active growth zones, such as young leaves (Roitsch and Gonzalez 2004). Neutral invertase, in turn, acts to control cell hexose levels in lower metabolic rate tissues compared with meristematic tissues (Sonnewald et al. 1997; Sturm 1999).

Referring to sugar level alterations, reducing sugars were increased only until stage C, with no differences among the genotypes and thereafter were maintained stable (Fig. 9).

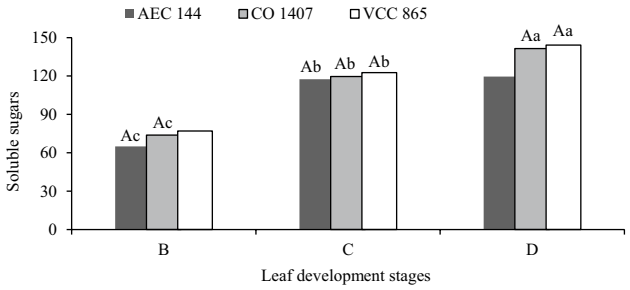

Fig. 10 Soluble sugar content at different leaf development stages in *Eucalyptus* young plants, genotypes AEC 144, CO 1407 and VCC 865. Data are expressed as µmol soluble sugar g^{-1} fresh weight. Within each leaf development stage, mean values followed by the same capital letter indicate that genotypes are not different. Within each genotype, mean values followed by different lowercase letters indicate significant variation among the leaf development stages. Mean values were compared using Tukey's test ($p < 0.05$)

Soluble sugars, in turn, were increased at all development stages, also with no differences among the genotypes (Fig. 10). According to Takayanagi and Yokotsuka (1997), reducing sugars increase in leaf maturation is due to the high assimilate transport rate which is required for structural sugar synthesis, such as cellulose, pectin and hemicelluloses. On the other hand, referring to rubber tree leaf development, Miguel et al. (2007) attributed higher photosynthesis rate at stage D to the higher photochemical efficiency in photosystem II and carboxylation efficiency, which provide sugar content increase. Based on these previous studies, we consider that the reducing and soluble sugar increase in sink-to-source leaf transition can be attributed to higher photosynthesis rates.

Conclusion

The duration of total leaf development, in young *Eucalyptus* plants, genotypes AEC 144, CO 1407 and VCC 865, under partially controlled conditions and without environmental stress, ranges from 44 to 49 days. Four stages—hereinafter named A, B, C and D—are now established to represent chronologically noticeable changes in leaf ontogeny, such as size, shape and color.

Stage A (12–15 days) is the beginning of leaf ontogeny and starts with the bud arising in the axis stem, which forms a petiole similar to a pin. At the end of longitudinal petiole growth, leaflet opening occurs, when the leaf blade starts to be established. Petioles can be totally green, but usually also exhibit reddish or brownish tones in veins, with greenish tones in the central region. The main features at stage B (10 days) are a leaf blade expansion, which is largest in length than in width, and a color change, when original reddish or brownish tones begin to be replaced by green color. At stage C (8–10 days), intense leaf blade growth occurs in the longitudinal direction, concomitant with prominent advent of veins. The leaf margin still shows reddish and brownish tones, but the green color becomes widely predominant. At stage D (14 days), the leaves become fully green, thicker, more consistent and completely expanded. The final leaf dimension in each genotype shows longer appearance in CO 1407, wider in AEC 144 and intermediate in VCC 865.

The physiological features, such as dry mass, leaf area, photosynthetic pigment content and photosynthesis rates, are increased throughout leaf development. In the early development stages, the mean values of these features are similar, on comparing with the genotypes, but from stage C, however, they become larger in the AEC 144 genotype than in the CO 1407, which in turn are larger than in VCC 865. Decrease in sucrose hydrolysis by invertases and increase in reducing and soluble sugar content are also found during leaf ontogeny. These biochemical and metabolic changes can be interpreted as evidences of sink-to-source leaf transition, which is consolidated from stage C.

Author contribution statement Authors' contribution statement Conceptualization: PARC, JRJS. Plant cultivation, daily photographing and data collection: JRJS, TCL. Laboratory chemical analyses: JRJS, RAAB, MPB, MOS, TCL. Data analysis and interpretation: JRJS, PARC. Original writing and english language translation: PARC, JRJS.

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