



Physiological and Ultrastructural Characteristics of Leaves of Eucalyptus Clones Destined for Matrix Selection

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Authors' contributions

This work was carried out in collaboration between all authors. Author LAML designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JVRP, GBR, KBR, GGS, HAAS, GHOD and LBP managed the analyses of the study. Author PAMF managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: In order to characterize Eucalyptus species for diverse purposes, it is necessary to analyze it in an ultrastructural and physiological way, aiming a faster and more precise selection of the possible properties and utilities of the species.

Objective: The objective was to observe the physiological and ultrastructural characteristics of leaves of eucalyptus clones destined for matrix selection.

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Materials and Methods: The following clones were used: E54, E20, E24, E45, AEC063 and E16. The following variables were evaluated: CO₂ assimilation rate expressed by area (A – $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration (E – $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), stomatal conductance (GS – $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), internal CO₂ concentration in the substomata chamber (Ci – $\mu\text{mol mol}^{-1}$), phloem diameter of leaf (PDL), xylem diameter of leaf (XDL), thickness of palisade parenchyma (PP), abaxial (ABET) and adaxial (ADET) surface thickness, inferior face of stomata functionality (IFFS) and stomata density (SD).
Results: The physiological and morphological variations were observed among the clones tested.
Conclusion: Clone E16 presented greater leaf transpiration, stomata conductance, the rate of photosynthesis and efficient water use, physiological, palisade parenchyma and stomata functionality. Clone E16 presented lower Adaxial epidermis thickness (AD) and Abaxial epidermal thickness (AB).

Keywords: *Eucalyptus grandis*; genetical enhancement; plant morphology; stomata; xylem.

1. INTRODUCTION

Nowadays, eucalyptus has been used as raw material in different industrial sectors, such as cellulose, paper and coal. However, use of its woods is increasing in civil construction as well as the use of its leaves to the extraction of essential oils [1]. Eucalyptus is also used in reforestation projects of degraded lands, once it has fast growth and good adaptation to different environments so that it benefits the preservations of reminiscent native forests [2,3,4].

Morphoanatomical studies of leaves have great importance in order to know characteristics of species, or clones, set to the obtainment of matrices trees in Eucalyptus breeding programs, once they are linked to the enhancement of biomass of the plants as well as to its adaptability in the use of available sources [4,5]. However, unique cultivar's and species' leaves may display the ultrastructural difference between leaves from the upper and lower area of the treetop, due to the intensity of light.

Regarding the morphology, one of the features displayed by leaves under full sun is its suitability for relation between surface and volume, since they can show a minor leaf area within a wide thickness, showing, that way, denser and heavier leaves per unit as compared to leaves cultivated in the shadows [6,7].

There is a lack of studies about ultrastructural characteristics of leaves of eucalyptus, which makes necessary new researches in order to evidence them to a better use of genetic material in vegetal breeding programs. According to [8], correlations between leaves with wood quality must be wary, since biotic and abiotic factors may influence the changing activity, then, increases the

heterogeneity of the wood and the distribution of xylem vases.

Understanding of clones' physiological parameters in plants breeding programs is a key role in decisions-making process regarding the choice of the best matrix to be used. These variations in its physiology may be a phenotypic response of the progeny to the environment in which is inserted [4]. That way, their characterisation makes a decisive tool in decisions-making on the choice of these matrices, due to the plasticity of their physiological responses to biotic and abiotic environmental stresses.

Therefore, the objective was to observe the physiological and ultrastructural characteristics of eucalyptus clones's leaves destined for matrix selection.

2. MATERIALS AND METHODS

2.1 Growth Conditions and Experimental Design

The experiment was carried out in September 2017 at Integrated College Stella Maris (FISMA), located in the city of Andradina, state of São Paulo, Brazil. The treatments were composed of six eucalyptus (*Eucalyptus grandis*) matrices: E54, E20, E24, E45, AEC063 and E16 and with 4 replicates.

The matrices were obtained in the varietal nursery of the company Eldorado Brasil Celulose Plants Nursery located in Andradina and were about 5 years old. They were grown in pots with a capacity of 100 litres containing soil. During four years, the matrices were fertilised [9], irrigated periodically until the field capacity was

reached, and all necessary cultural practices were done.

2.2 Gas Exchange Parameters

Gas exchange was evaluated via non-destructive analyses using a portable gas exchange device (Infra-Red Gas Analyzer – IRGA, brand ADC BioScientific Ltd, model LC-Pro). The following parameters were set: CO₂ assimilation rate expressed by area ($A - \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration ($E - \text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), stomatal conductance ($GS - \text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and internal CO₂ concentration in the substomatal chamber ($C_i - \mu\text{mol mol}^{-1}$). The initial conditions imposed for the measurements were 1000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ of photosynthetically active radiation (PAR), provided by LED lamps, 380 ppm of CO₂, under a 28 °C chamber temperature.

2.3 Ultrastructural Leaf

Fragments of the main leaves, on their middle part, also were selected. The samples were transported to Laboratory of Vegetal Morphophysiology and Forages at College of Agricultural and Technological Sciences – São Paulo State University. The collected material was immersed in F.A.A. 70 (formaldehyde 37%, acetic acid and 70% ethanol in the ratio of 1.0: 1.0: 18.0 - V / V). Twenty four hours after, the fragments were washed and stored in 70% ethanol until the date of the analyses, as described by [10]. All fragments of plant tissues were treated with the pertinent procedures for dehydration, diaphanization, inclusion and embedding. By using a microtome Leica that contains steel razors, eight- μm transversal sections were done in each embedded fragment.

The first transversal sections without damage caused by the cut of plants tissues were chosen for preparation of the histological slides. These sections were fixed with patches (albumin), were tinted with safranin with a 1% ratio, and were set in microscope and glass slides with Entellan® patch [10].

All slides were observed with an Olympus optical microscope, model BX 43, with an attached camera to perform the photos of the cuts. Pictures were used to measure anatomic parameters through the software cellSens Standart, that was calibrated with a microscopic ruler in the same gains.

By using transversal sections, the following ultrastructural variables were measured: Phloem Diameter of Leaf (PDL), Xylem Diameter of Leaf (XDL), Thickness of Palisade Parenchyma (PP), Abaxial (ABET) and Adaxial (ADET) Surface Thickness. The lower or abaxial epidermal impression of the fragments collected using cyanoacrylate ester was used to determine: Inferior Face of Stomata Functionality (IFFS) [11], and Stomata Density (SD). Ten measurements were done for all characteristics in each microscope slide. Plots were represented by average value obtained in each characteristic.

2.4 Statistical Analysis

In all of the datasets considered, the normality of the data was analysed using the Anderson-Darling test and homoscedasticity was analysed with the variance equation test (or Levene's test). All variables were submitted to the F test ($p < 0.05$) and the means were compared using the Tukey test ($p < 0.05$) [12], by using Assistat 7.7 static software [13] system for Windows 7.0.

3. RESULTS

3.1 Gas Exchange Parameters

Fig. 1 shows average values of inner concentration of CO₂ ($C_i - \mu\text{mol mol}^{-1}$) in eucalyptus clones used to matrices formation.

Statistical difference was not observed ($p \geq 0.05$) between eucalyptus clones at an inner concentration of CO₂ on leaves, which presented a $263.19 \pm 14.74 \mu\text{mol mol}^{-1}$ average. However, it was detected a statistical difference between them ($p < 0.01$) on the variable leaves transpiration, highlighting E16 that presented approximate difference of 38.00% as compared with E54, which presented the best average.

Once again, E16 displayed a greater stomata conductance with a 40.54%-difference as compared with E54, which presented the lowest averages, as Fig. 1C shows. To the variable rate of photosynthesis, E16 matrix highlights as compared to others materials, by showing a 32.20%-difference from E45, which showed the best result. (Fig. 1D). Regarding the variable efficiency in the use of water, it was not observed a static difference ($p \geq 0.05$) among the eucalyptus clones that displayed a 1.49 ± 0.23 general average (Fig. 1E).

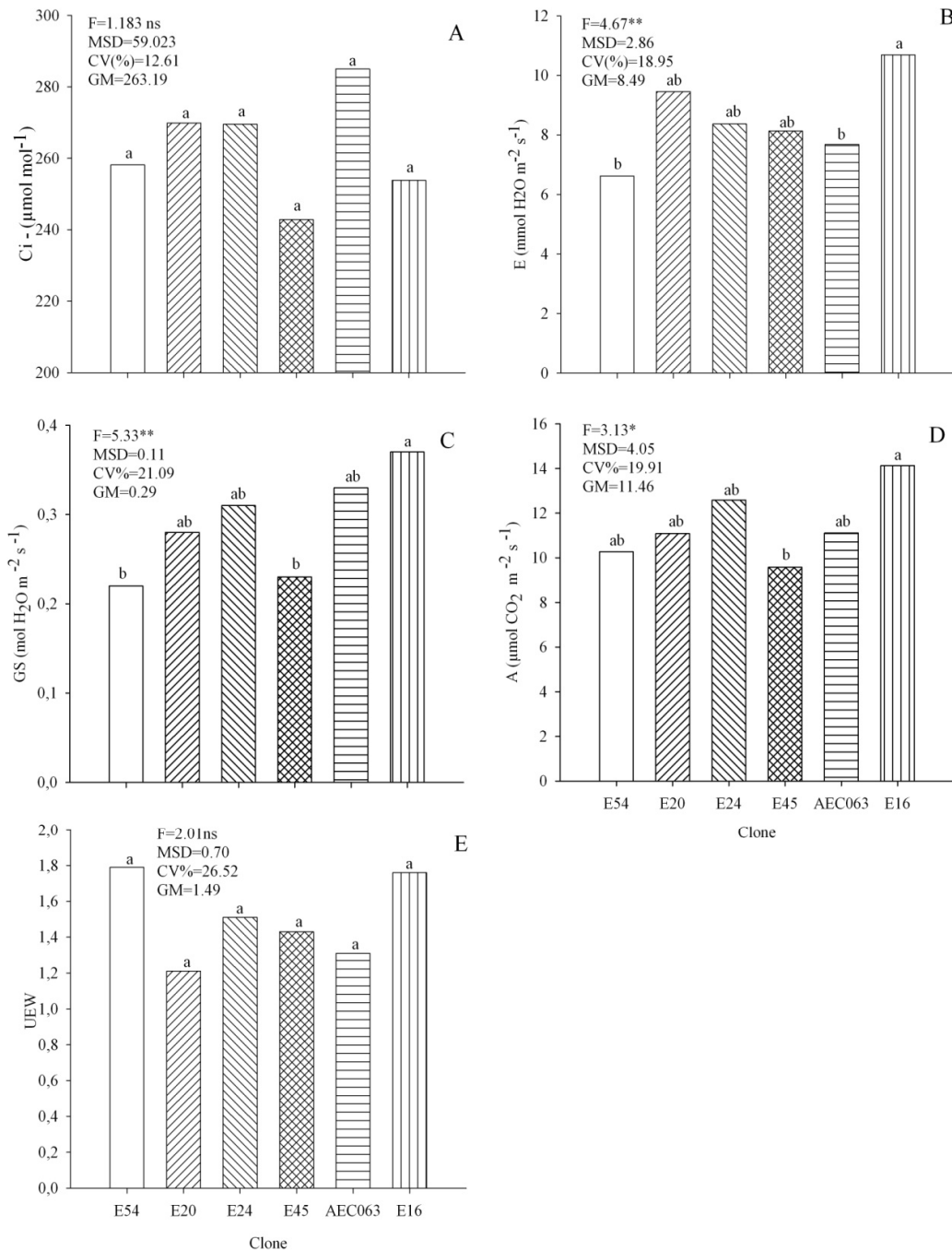


Fig. 1. Mean values of internal CO₂ concentration (Ci), Leaf transpiration (E), Stomatal conductance (Gs), Photosynthesis rate (A) and Efficiency of water use (UEW) of eucalyptus clones destined to the formation of matrices. Andradina, 2018. MSD: Minimum significant difference, CV: Coefficient of variation, GM: General mean and F: value of F calculated in the analysis of variance, **significant at the 1% probability level (p<0.01), *significant at the 5% probability level (0.01=<p<0.05), ns - not significant (p>=0.05). The averages in the column followed by the same letter do not differ statistically from each other. The Tukey test was applied at a 5% probability level

3.2 Ultrastructural Leaf

Significant difference was not observed ($p>=0.05$) among the eucalyptus clones that displayed a 10.77 ± 0.76 general average on

xylem diameter of leaf (Fig. 2A), as well as to the variable phloem diameter of leaf they do not show statistic difference ($p>=0.05$) obtaining a 2.91 ± 0.20 general average, as Fig. 2B shows.

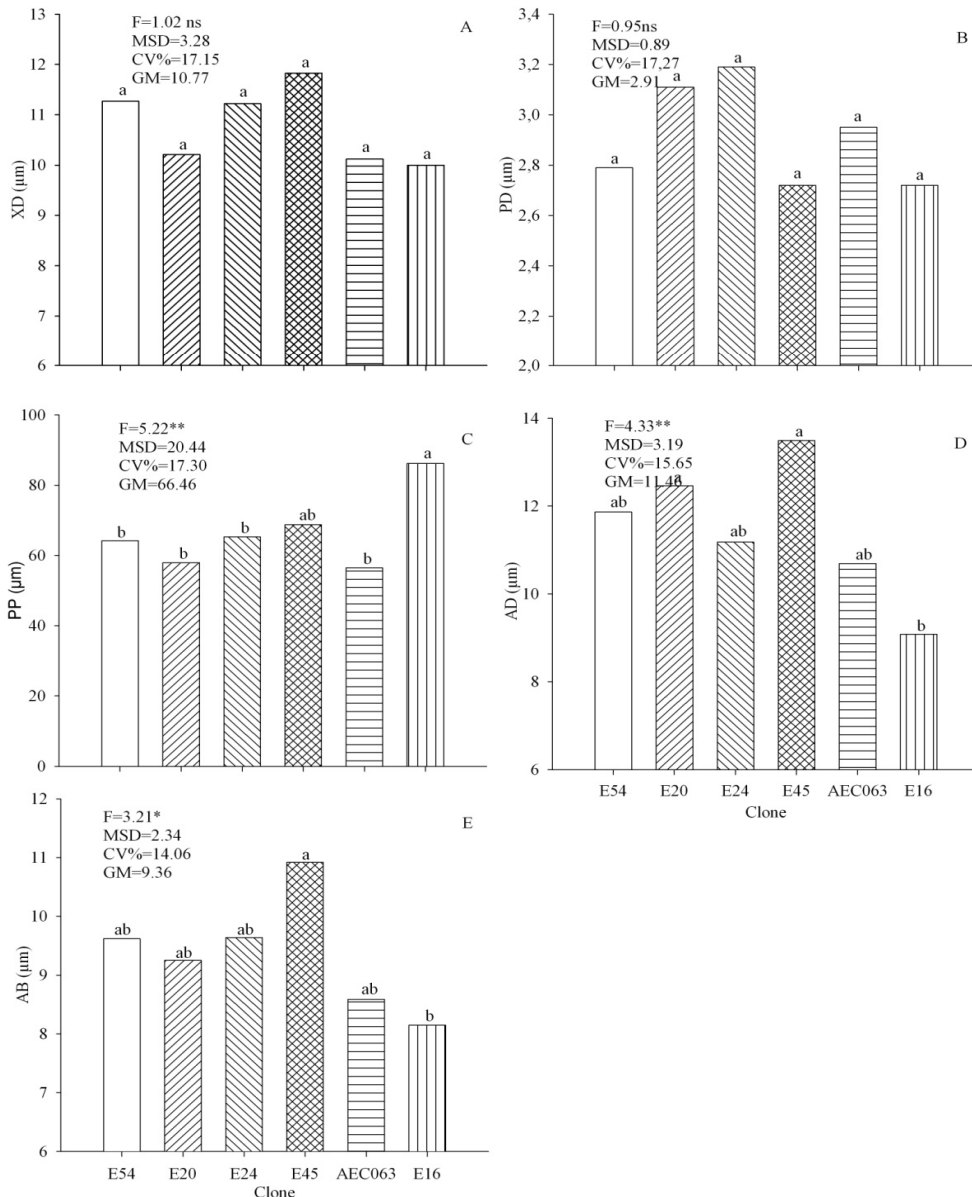


Fig. 2. Mean values of xylem diameter (XD), Phloem diameter (PD), Thickness of palisade parenchyma (PP), Adaxial epidermis thickness (AD) and Abaxial epidermal thickness (AB). MSD: Minimum significant difference, CV: Coefficient of variation, GM: General mean and F: value of F calculated in the analysis of variance, **significant at the 1% probability level ($p<0.01$), *significant at the 5% probability level ($0.01=<p<0.05$), ns - not significant ($p>=0.05$). The averages in the column followed by the same letter do not differ statistically from each other. The Tukey test was applied at a 5% probability level

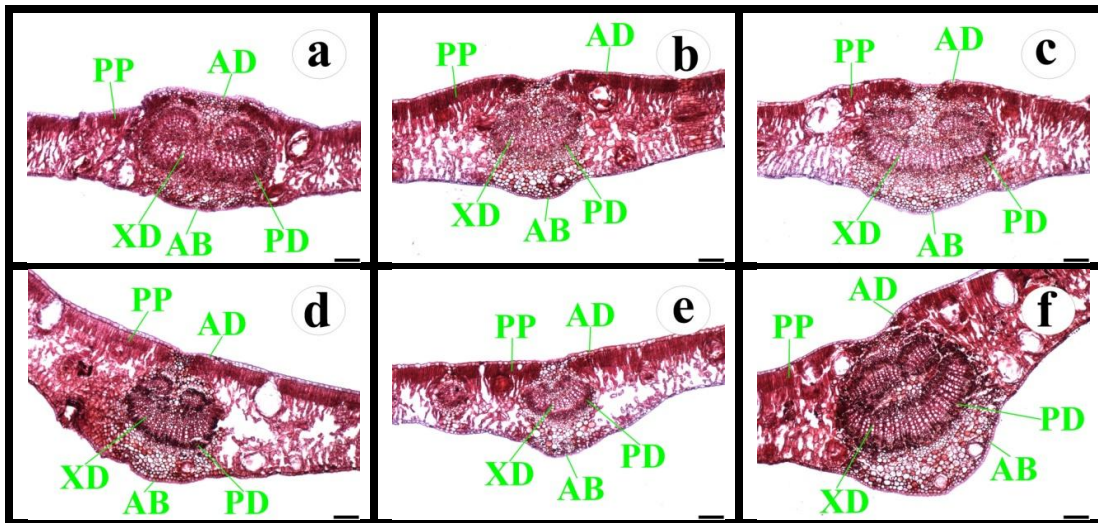


Fig. 3. Ultrastructural leaf of eucalyptus matrices (*Eucalyptus grandis*). a- Clone E54, b- Clone E20, c- Clone E24, d- Clone E45, e- Clone AEC063 e f- Clone E16. and abaxial (AB) and adaxial (AD) surface thickness. Bar= 20 μ m, 200X magnification

However, it was observed that E16 clone statically differs from the others materials in the variable thickness of palisade parenchyma, which presented an approximated 32.82% average upper value as compared to E20, which presented the lowest average value (Fig. 2C). This difference is noticeable when the leaf ultrastructures of the eucalyptus clones are observed and compared in Fig. 3A and F.

Regarding the variable abaxial and adaxial surface thickness, E45 statically differs from the others materials, being respectively 32.69% and 25.36% thicker than E16, which presented the lowest average value, as described on Fig. 2D and E. However, it was not observed statistical difference on the matrices regarding stomata density ($p \geq 0.05$), as Fig. 4A shows. Fig. 5 shows similar stomata density in different clones.

However, to the variable stomata functionality, E16 statically differs from others clones, being 37.15% higher as compared to E54, which presented the lowest average value, as described on Fig. 4B.

4. DISCUSSION

Inner concentration of cellular carbon is fundamental to a good fixation of dry mass during vegetal development, this variation may occur in different positions of the plant stem or even at concentration of nitrogen used on catalytic reactions of the RuBisCo molecule [14,

15, 16], present in cells of the palisade parenchyma, by having this property, this tissue can present greater development, as Fig. 2C shows.

E16 displayed greater leaf transpiration, which may be a limiting factor as cultivated in environments with water deficiency at some point of the year or lands with sandy soils that has a good infiltration. With the elevation of leaf transpiration, the needs of water availability in soil-plant-atmosphere system were increased [5, 17]. Leaf transpiration may be associated to a thickness of abaxial and adaxial epidermis, where E16 showed the lowest averages, as Fig. 2D and E show, this characteristic may be an important factor in the decision-making process of the choice of material to breeding programs that aim more resistant plants as exposed to water stress.

Stomata conductance may be associated to density or stomata functionality. Since stomata density did not display a statistical difference among the clones, as Fig. 4A and 5 show, this density may have not contributed significantly with conductance and leaf transpiration but may have contributed with stomata functionality, because E16 displayed a higher average of functionality, which, in its turn, a greater transpiration and conductance, as Fig. 1B and C show. Stomata functionality represents its opening through horizontal and vertical diameters, and, as it increases, the water loss may have been potentialized [11, 18].

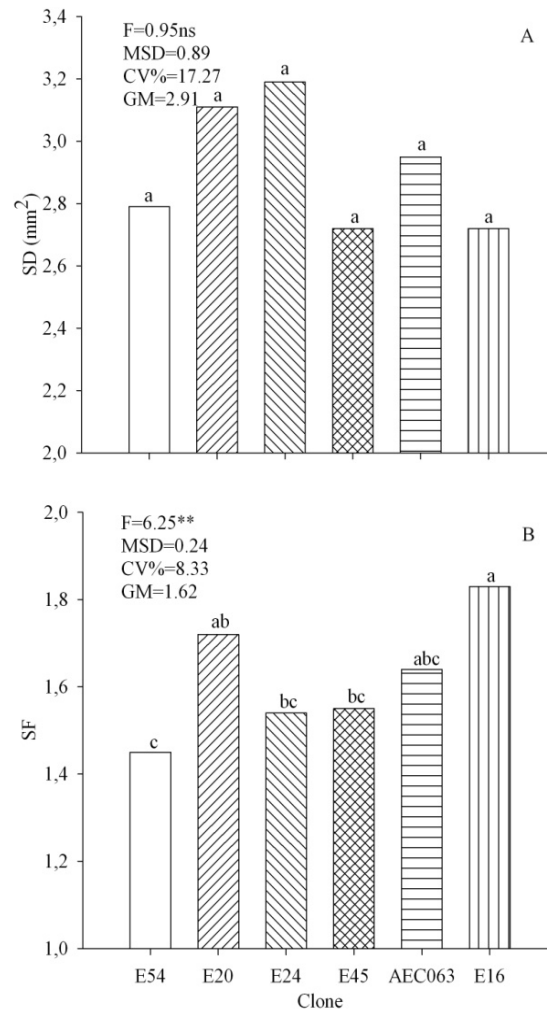


Fig. 4. Mean values of stomatal density and stomatal functionality of eucalyptus clones destined to the formation of matrices. Andradina, 2018. MSD: Minimum significant difference, CV: Coefficient of variation, GM: General mean and F: value of F calculated in the analysis of variance, **significant at the 1% probability level ($p < 0.01$), *significant at the 5% probability level ($0.01 \leq p < 0.05$), ns - not significant ($p \geq 0.05$). The averages in the column followed by the same letter do not differ statistically from each other. The Tukey test was applied at a 5% probability level

A greater photosynthesis rate can imply a bigger carbon fixation on dry mass, and, consequently, a bigger yield of wood [19]. E16 highlights among the studied clones in this variable, so that it is expected that E16, as submitted to adaptation stages in production areas, will present the same response, as well as its characteristics, will be transmitted to its progenies in breeding programs. This photosynthesis rate may be directly linked to the size of the cells of the palisade parenchyma, corroborating with the results that Fig. 2C shows. Palisade parenchyma

is the tissue that presents a greater density of active photosynthetic organelles, which, in its turn, presents bigger cells, as Fig. 3 shows.

With of significant difference in the photosynthesis rate, it was expected the same results in the efficient use of water, which did not occur, as Fig. 1E shows. The difference in the efficient use of water among the clones cultivated under water stress, related to light variation, which influenced linearly the development of eucalyptus [20].

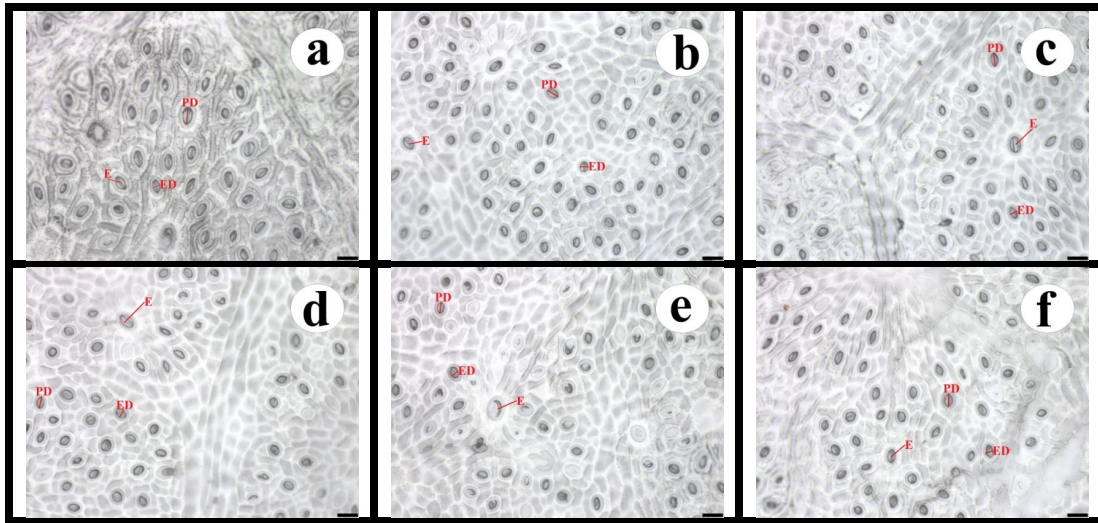


Fig. 5. Lower or abaxial epidermal impression of the leaf fragments of eucalyptus (*Eucalyptus grandis*). E- Stomata, PD- Diameter polar and ED- Equatorial diameter of stomata of eucalyptus of matrices. Bar= 10 μ m, 400X magnification

Similarly, it was not observed a statistical difference among the studied material on the phloem and xylem diameter, once it was expected a greater xylem diameter in E16, which is the responsible tissue by the water transportation from soil to leaves of the upper Eucalyptus's area, which could have been influenced by leaf transpiration and conductance [21].

It is noticeable the necessity of deeply knowing the tissue's plasticity and its relation with vegetal physiology, since, it allows a more accurate decision-making process regarding the choice of the materials that will be used in breeding programs of Eucalyptus [22].

5. CONCLUSION

Clone E16 presented greater leaf transpiration, stomatal conductance, rate of photosynthesis and efficient water use, physiological, palisade parenchyma and stomatal functionality.

Clone E16 presented lower Adaxial epidermis thickness (AD) and Abaxial epidermal thickness (AB).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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