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Research article

The ideal percentage of K substitution by Na in *Eucalyptus* seedlings: Evidences from leaf carbon isotopic composition, leaf gas exchanges and plant growth

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ABSTRACT

Potassium (K) is the most required macronutrient by *Eucalyptus*, while sodium (Na) can partially substitute some physiological functions of K and have a positive response on plant growth in K-depleted tropical soils. However, the right percentage of K substitution by Na is not yet known for *Eucalyptus* seedlings, since a few experiments have only compared treatments receiving K or Na. This study evaluated five levels of Na supply (0, 0.45, 0.90, 1.35 and 1.80 mM) as substitution for K in *Eucalyptus* seedlings grown in nutrient solution. Plants growth, biomass, K-nutritional status, leaf gas exchange, leaf carbon isotopic composition ($\delta^{13}C$ ‰), leaf water potential (Ψ_w), leaf area (LA), stomatal density (SD) and water use efficiency (WUE) were measured. The highest total biomass yield was achieved by the Na estimated rate of 0.25 mM, corresponding to a leaf K: Na ratio of 3.41, and having the lowest $\delta^{13}C$ values. Conversely, the highest Na rate (1.8 mM) induced K deficiency symptoms, lower growth, reduced total dry matter yield, leaf gas exchange, LA, SD and a higher $\delta^{13}C$, which presented a trend to an inverse correlation with CO_2 assimilation rate (*A*), WUE and shoot dry matter. Collectively, our results conclude that substitution of 25% of K by Na (0.45 mM of Na) provided significant gains in nutritional status and positive plant physiological responses by increasing WUE, stomatal diffusion, and by augmenting CO_2 uptake efficiency. This nutritional management can therefore be an alternative option to optimize yields and resource use efficiencies in *Eucalyptus* cultivation.

1. Introduction

Recent population growth estimates indicate that world population will reach 9.1 billion people by 2050, which will boost wood demand and require planting an additional 250 million ha of planted forests worldwide (IBA, 2017). This challenge, in turn, necessitates new silvicultural techniques associated with land use, water and nutrient resources in order to improve plant yield (IBA, 2017). In Brazil, *Eucalyptus* is the most widely planted forest genus, in which *E. urophylla* × *E. grandis* hybrid has been widely used (represents up than 70% of hybrid plantation) in regions with water stress and infertile soil (Assis et al., 2015; Gonçalves, 2010; Goncalves et al., 2013). Because of this, fertilization becomes essential to plants in order to achieve high productivity, as already reported in *Eucalyptus* growth responses to nitrogen (N), phosphorous (P) and potassium (K) (Melo et al., 2016).

Sodium (Na) can partially replace K physiological functions that operate in plant vacuole, such as osmotic control and enzymatic reactions, as they are essential to enable adenosine triphosphatase (ATPases) (Wakeel et al., 2011). In addition, Na improves plant water balance and water use efficiency (WUE) by modifying stomatal control

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Abbreviations: *A*, CO_2 assimilation rate; *Ci*, internal carbon dioxide concentration; LA, leaf area; $\delta^{13}C$ ‰, leaf carbon isotopic composition; Ψ_w, leaf water potential; K, potassium; PAR, photosynthetically active radiation; Na, sodium; SLA, specific leaf area; g_s, stomatal conductance; SD, stomatal density; *E*, transpiration rate; WUE, water use efficiency

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of plant transpiration rate (Subarao et al., 2003; Marschner, 2012), and by contributing to the maintenance of cell turgor and expansion (Wakeel et al., 2011). With Na application, C_3 plants suffer large changes in carbon allocation, replacing K as active osmotically solute and stimulating cell elongation (Britto and Kronzucker, 2015; Cui et al., 2018). As K^+ is the main electrolyte for plants, Na^+ and K^+ are similar both chemically and structurally (Kaur et al., 2016). This may happen since Na has a radius of 0.358 nm, whereas K has 0.331 nm (Marschner, 2012). However, Na effects on water balance are not well studied in any species despite the well-recognized importance of K (Coskun and Britto, 2013; Gattward et al., 2012). The differences in the growth responses of halophytes or natrophilic (Na includes, with moderate to high salt tolerance) and natrophobic or glycophytes (Na excluders, with low salt tolerance) species are related to differences in uptake and in root-to-shoot Na-translocation (Kronzucker and Britto, 2011; Kronzucker et al., 2013; Pilon-Smits et al., 2009; Subarao et al., 2003).

Positive responses by the small NaCl supply in K-depleted soils were found in coconut (Bonneau et al., 1997), ryegrass (Huhtanen et al., 2000), cotton (Zhang et al., 2006), sugar beet (Wakeel et al., 2010), and cocoa (Gattward et al., 2012). In these studies, NaCl supply increased the quality and yield of crops, mainly by replacing K^+ as co-factors for certain enzymes (Kaur et al., 2016), and by changes in cell water relation, improving the stomata control, reducing transpiration rate, thus, increasing the water use efficiency (WUE) as well as augmenting CO_2 uptake efficiency (Pilon-Smits et al., 2009; Subarao et al., 2003). Positive response to NaCl application in *E. grandis* growth in K-depleted Brazilian soils has also been observed (Almeida et al., 2010; Epron and Laclau, 2012; Battie-Laclau and Laclau, 2013; 2014). In addition, K substitution by Na may be important in soils that are high K 'fixers' (Wakeel et al., 2010). Finally, another great benefit of K replacement by Na in fertilization is the relatively lower cost of NaCl compared to potassium chloride (KCl) (Battie-Laclau and Laclau, 2013; Marschner, 2012), bringing greater profitability to the forestry sector and a possible strategy to increase K efficiency.

In the field of plant nutrition and physiology, even though some studies have been done on the substitution of K^+ by Na^+ in crops, it is still poorly documented in trees (Battie-Laclau and Laclau, 2013; 2014; Gattward et al., 2012). In this regard, Na^+ has the potential to partially replace K^+ for some of nonspecific functions, like osmotic adjustments, turgor pressure and cell expansion (Wakeel et al., 2011). In some plants, Na supply is able to prevent or reduce considerably the occurrence of K^+ deficiency symptoms under limited K^+ supply (Battie-Laclau and Laclau, 2013; 2014). Furthermore, little is understood about the physiological mechanisms that improve *Eucalyptus* growth when low amounts of Na are applied in weathered soils with low K-available levels, especially the effects of Na supply on leaf area components as well as on the diffusional and biochemical limitations to photosynthesis in *Eucalyptus* leaves (Battie-Laclau and Laclau, 2013; 2014), where gas exchange occurs.

In the field of plant nutritional genomics and molecular biology, it has been stated that Na^+ supply at low levels is beneficial in the presence of low K^+ availability. At high Na^+ levels, low-affinity fluxes may catalyze Na^+ transference via K^+ specific ion channels such as AKT1 (inward rectifying K^+ channel), HKT (high-affinity K^+ channels), non-selective ion channels (NSCC), and outward rectifying K^+ channels (ORC). However, in non-halophytes plants such as *Eucalyptus*, there is a Na^+-H^+ antiporter, SOS1 (salt overly sensitive), resulting in the efflux of Na^+ ions (Britto and Kronzucker, 2015; Kaur et al., 2016; Kronzucker and Britto, 2011; Maathuis, 2014). In response to K deficiency and water stress, plant redistributes leaf ABA and upregulate hormone synthesis in roots, leading to an increase in ABA accumulation in the apoplast of stomatal guard cells. The elevated ABA triggers a chain of events in guard cells, causing stomatal closure and thus preventing water loss (Pei and Kuchitsu, 2005). Plant stress events associated with ABA-induced stomatal closure in guard cells include, sequentially, the production of reactive oxygen species (ROS), increases in cytosolic free Ca^{2+} levels, activation of anion channels, membrane potential depolarization, cytosolic alkalinization, inhibition of K^+ influx channels, and promotion of K^+ efflux channels (Pei and Kuchitsu, 2005). Likewise, it has been stated that high Na^+ levels stimulate abscisic acid (ABA) production, leading to a leaf senescence and chlorophyll degradation (Kaur et al., 2016; Maathuis, 2014; Silva and Gerós, 2009).

Besides these myriad responses, Na supply can also be toxic for plants depending on levels applied (Maathuis, 2014), which demands caution in its use and also indicates the need for specific studies on *Eucalyptus* in order to achieve a better K:Na ratio on plant status. Furthermore, it is worth to mention that the ideal percentage of K substitution by Na for *Eucalyptus* seedlings is not yet known, since the studies carried out with Na and *Eucalyptus* commented above, focused in salt tolerance at high saline levels and the complete substitution of KCl by NaCl application, without mentioning the positive effects of a variable supply of Na in combination with K (Sette et al., 2013). In addition, it is important to bear in mind that essential-to-lethal range for Na supply to non-halophytes genus like *Eucalyptus* is probably somewhat narrow. Likewise, the physiological effect of Na supply at optimal level deserves more attention with regard to using it to fertilize *Eucalyptus* trees in order to fulfill plant's nutritional requirement under K-depleted infertile soils, as well as to boost plant growth under stress conditions.

Here, we studied the effects of ideal Na percentage of K substitution on important physiological parameters involved in leaf gas exchange (photosynthesis, stomatal conductance, stomatal density, water loss, $\delta^{13}C$ (‰) isotope composition), plant growth (specific leaf area, root/shoot ratio, plant biomass yield), and leaf K and Na concentrations. The objectives were: 1) to gain insight into the K and Na nutritional interactive effects on plant growth and leaf gas exchanges in *Eucalyptus* seedlings, and 2) to determine the ideal percentage of K replacement by Na.

2. Material and methods

2.1. Plant growth conditions and experimental design

The experiment was carried out in a greenhouse at the Center for Nuclear Energy in Agriculture (CENA-USP) in Piracicaba, São Paulo State, Brazil from November 2014 until February 2015. The temperature in the greenhouse during plant growth ranged between 24.2 °C (minimum) and 36.1 °C (maximum), and averaged 31.4 °C. The average air relative humidity was 65%, and the maximum photosynthetic photon flux density (sunlight) was approximately 1,600 $\mu mol\, m^{-2}\, s^{-1}$.

A total of 40 plants of the *Eucalyptus* clone (*E. urophylla* × *E. grandis* hybrid - elite clone widely cultivated in Brazil due to its importance in areas with occurrence of soil water deficit and infertile soils, see Assis et al., 2015; Gonçalves, 2010; Goncalves et al., 2013) with approximately 90 days and 30 cm of height, arrived in plastic tubes, had the substrate removed from their roots with tap water, and were then transplanted to a collective tray (10 L) containing Clark's nutrient solution (Clark, 1975), being cultivated for 30 days, representing the acclimatization stage in the nutrient solution. In order to avoid osmotic shock, the concentration of the nutrient solution in which the seedlings were grown in was changed weekly, gradually increased by 25, 50, 75, and 100% of ionic strength of the medium. Furthermore, plants were kept under permanent aeration of the nutrient solution, which it was replaced every 7 days. The solution was oxygenated throughout the entire experiment through plastic tubes connected to an air compressor. At the end of acclimatization stage, homogeneous plants were selected according to height and collar diameter, and transferred to individual plastic pots (3 L) where treatments were applied for 63 days.

The experiment replaced the original K rate of Clark's nutrient solution (1.80 mM of K, as KCl) by increasing the Na applied percentage (as NaCl), which represented a substitution of 0/100, 25/75, 50/50, 75/25 and 100/0 (% Na/% K). Thus, the treatments consisted of five combinations of Na and K application rates (0/1.80, 0.45/1.35, 0.90/0.90, 1.35/0.45 and 1.80/0 mM of Na/mM of K). The higher applied Na rate (1.80 mM), with no K supply (0 mM), represented the highest K-deficiency stress treatment to plants. The experiment was installed in a randomized block design with four replications, making 20 experimental units with one plant in each. The pH of the nutrient solution was measured, reaching values of around 5.5 during its exchange.

2.2. Plant growth, leaf gas exchange parameters and leaf water potential

A graduated ruler (in cm) and a digital caliper (in mm) were used to compute the weekly growth in height and collar diameter, respectively. Before harvesting, the youngest fully expanded leaf of each plant was used to evaluate the gas exchange in the morning (7–11 a.m.) using an infrared gas analyser (LI-6400; LI-COR Inc., Lincoln, NE, USA) under a photosynthetic photon flux density of 1,800 μmol m^{-2} s^{-1} and air CO_2 concentration of 380 μmol mol^{-1}, at a leaf temperature of between 21 and 25 °C, in accordance with Santos et al. (2017, 2018), and Reis et al. (2018).

The mean leaf temperature during the measurement was 28.9 °C. CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration rate (E), and the relation between internal and external carbon dioxide (CO_2) concentration (C_i and C_a respectively) were measured. From this, it was possible to calculate the instantaneous water use efficiency (WUE) level in the leaf by dividing values of A by E (Battie-Laclau and Laclau, 2013; Martineau et al., 2017). The same type of leaves assessed for gas exchange were used to measure the leaf water potential (Ψ_w) with a Scholander pressure chamber (Model 610, PMS® Instrumental Company) at dawn (5 a.m.) and at noon (12 p.m.) (Turner, 1981).

2.3. Stomatal density and leaf area

Stomatal density (SD; stomates mm^{-2}) measurements were made on a subset of leaves using a rapid imprinting technique, which allowed us to reliably score hundreds of stomata at the same time. Four leaves were collected in the middle region of the canopy per plant (one on each side, considering the plant with four quadrants) and the abaxial leaf surface was printed onto a glass slide. The sampled foliar region was pressed for 10 s against a drop of Superglue (brand of glue) on the glass slide. Then, using an optical microscope (ZEISS, Scope.A1, Carl Zeiss Inc., Thornwood, NY, USA) with a 10× objective magnification and a digital camera attached, the leaves were photographed using three random fields of 0.116 mm^2 each. Stomatal counting was done using the ImageJ program (https://imagej.nih.gov/ij/) with 150× zoom, and the average SD was calculated (Segatto et al., 2004).

After 90 days of cultivation in the nutrient solution (days after onset of treatments), the plants had their leaves sprayed with deionized water to remove dust, then harvested and their leaves, stems, branches and roots separated. All leaves previously used, including those for SD analysis, were always kept in ice to avoid dehydration until taken to the laboratory for determination of leaf area (LA), which was obtained by passing all leaves through a digital area meter (LI-3100, Li-Cor, Inc., Lincoln, NE).

2.4. Dry mass production and plant chemical analysis

At harvest, the plants were separated into leaves, stems, branches and roots. The material was identified, packaged in paper bags, and dried, at 65 °C, for 72 hs in a forced-air oven, followed by the measurement of the dry mass. All samples (0.25 g DW) were microwave-assisted acid digested. A closed vessel microwave oven (ETHOS 1600©, Milestone, Italy) was used according to the following procedure: 250 mg of ground material was accurately weighed in the TFM vessels and then 6.0 mL of 65% v/v HNO_3 and 1.0 mL of 30% v/v H_2O_2 were added (Furlan et al., 2018; Lavres et al., 2019). Thereafter, the residual solutions were transferred to 25 mL volumetric flasks and the volume made up with high purity deionized water (resistivity 18.2 MΩ cm). The final solutions were analyzed by a radially viewed ICP-OES (Vista RL©, Varian, Australia). With the values of root and leaf K and Na concentration, it was possible to calculate the K:Na ratio in these parts of the plant. In addition, the optimal ratio in root and leaf that provided the higher dry matter production of *Eucalyptus* was correlated.

2.5. Leaf carbon isotope composition ($\delta^{13}C$ ‰)

The $\delta^{13}C$ isotope composition (‰) of the samples were determined using a mass spectrometer (ANCA-GSL Hydra 20–20 model, SERCON Co., Crewe, England) coupled to a carbon automatic analyzer (Barrie and Prosser, 1996). Values of $\delta^{13}C$ (‰) were calculated by Equation (1), described by Farquhar et al. (1982), Henderson et al. (1992) and Cernusak et al. (2013). With this data, the correlation between WUE and $\delta^{13}C$ was determined using:

$$\delta(‰) = (R_{(sample)} / R_{(standard)} - 1) \times 1000$$

where R is the isotope ratio of $^{13}C/^{12}C$.

2.6. Statistical analysis

The data were statistically analyzed using a SAS statistical package (SAS 9.0 Institute Inc. 2004) of an analysis of variance ($p < 0.05$) to test the effects of variable Na and K supply on measured variables. Linear, quadratic and square root regression models were utilized to describe the relations between Na supply and response variables, and the significant model ($p < 0.05$) with the highest determination coefficient (R^2) was selected. The variability of data was performed according to standard error and showed graphically using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Eucalyptus seedlings growth and dry matter yield

Weekly growth of *Eucalyptus* seedlings from the onset of Na application until plant harvest is shown in Fig. 2. Just 21 days after the treatment was applied, plant growth on the 0.45 mM of Na started to differentiate, keeping ahead of others until the experiment end; this was more evident for height than for collar diameter, achieving 108 cm plant^{-1} and 12 mm plant^{-1} in height and collar diameter, respectively (Fig. 2a and b). The exclusive supply of Na (1.8 mM of Na), decreased plants height (42%) and diameter (37%) respectively, compared to full supply of K (0 mM of Na).

Increased Na supply negatively affected root and shoot dry matter yield, as well as the root: shoot ratio (Table 1). *Eucalyptus* seedlings grown under 1.8 mM of Na showed a reduction of 72.5% of root dry matter yield compared to control – 1.8 mM of K (Table 1). The shoot and total dry matter decreased with increased Na concentration in the solution, respectively, by 76.5 and 76%, relative to the control treatment. The estimated Na concentration in the solution to give the maximum of total dry matter production of plants (68 g plant^{-1}) was 0.25 mM of Na and 1.55 mM of K, which means 14% of substitution. In addition, the root: shoot ratio decreased with increasing Na supply up to 1.35 mM. The root: shoot ratio of plants under 1.8 mM of Na was 13% higher than in plants under solely K supply, indicating reduced shoot growth compared to plants exposed to exclusive Na supply (Table 1).

3.2. Plant tissues K- and leaf Na-concentration and its correlation to g_s and with shoot dry matter yield of Eucalyptus seedlings

Potassium and Na-concentrations in plant tissues were affected by K

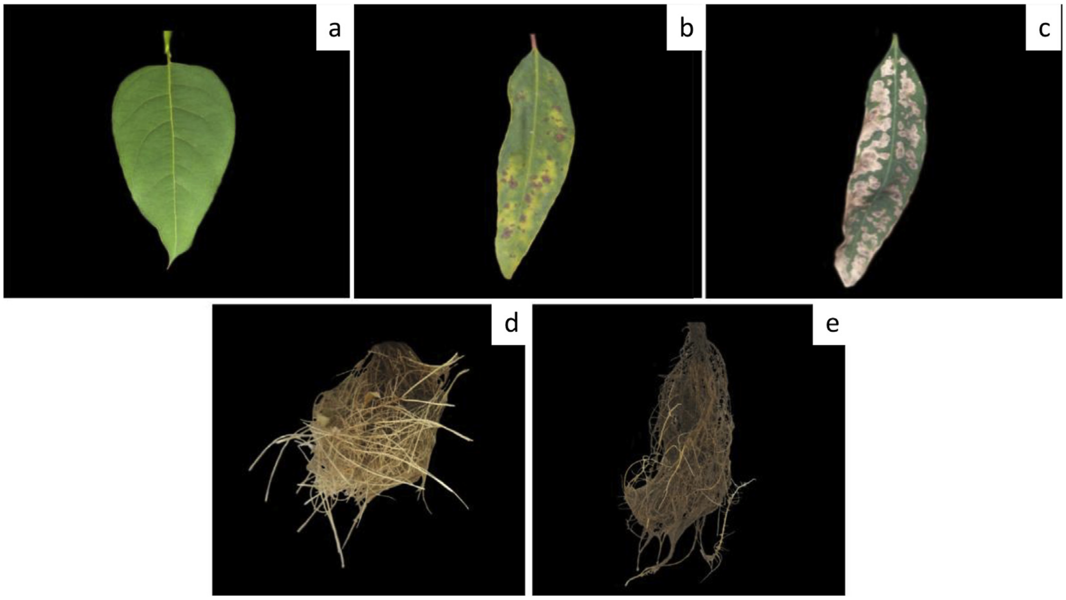

Fig. 1. Aspect of mature healthy leaves [K concentration of 13.5 g kg^{-1}] (a), visual symptom of chlorosis (b) and necrosis due to K deficiency [K concentration of 2.3 g kg^{-1}] (c) normal aspects of health root (d) and Na-hampered root (e) of *Eucalyptus* seedlings subjected to K replacement by Na in the nutrient solution.

and Na application in the nutrient solution (Fig. 2a and b). *Eucalyptus* seedlings grown without Na supply (0 mM of Na) showed K-concentration of 2.0 and 15 g kg^{-1} in the roots and leaves, respectively (Fig. 3a). On the other hand, plants subjected to the highest Na concentration in the solution showed Na-roots and Na-shoot concentration of 4 and 12 g kg^{-1}, respectively. In addition, Na concentration in plant roots increased up to Na rate of 0.9 mM in the nutrient solution (Fig. 3b). Conversely, Na concentration in the leaves increased 200% with Na application rate from 0 to 1.8 mM, indicating greater Na root-to-shoot translocation and accumulation in the shoot under higher Na supply (1.8 mM of Na). Furthermore, it was observed a positive correlation between leaf K concentration and g_s, as well as to leaf K concentration and total dry matter (Fig. 3d). Conversely, it was found a negative correlation among leaf Na concentration, g_s and total dry matter (Fig. 3e). The K:Na ratio followed the square root regression model, and according to the estimated rate of 0.25 mM of Na to achieve the maximum total dry mass yield of *Eucalyptus* seedlings (Table 1), the optimal leaf K:Na ratio of 3.41 was found (Fig. 3c).

3.3. Leaf gas exchange

The application of the Na rates in the nutrient solution significantly influenced the leaf gas exchange variables of *Eucalyptus* seedlings in most of the variables, except for C_i/C_a (Fig. 4). Overall, there was an increase in *A* (Fig. 4a), g_s (Fig. 4b), *E* (Fig. 4c) and WUE (Fig. 4e) of *Eucalyptus* seedlings when K was partially replaced by Na in the nutrient solution. Higher *A* was observed at the lowest Na rates of 0.45 and 0.90 mM, with values of around 13 μmol m^2 s^{-1} - corresponding to an increase of 7% relative to the control (Na rate of 0 mM). Otherwise, the highest Na rate (1.80 mM) provided the lowest values for *A*, g_s and *E*,

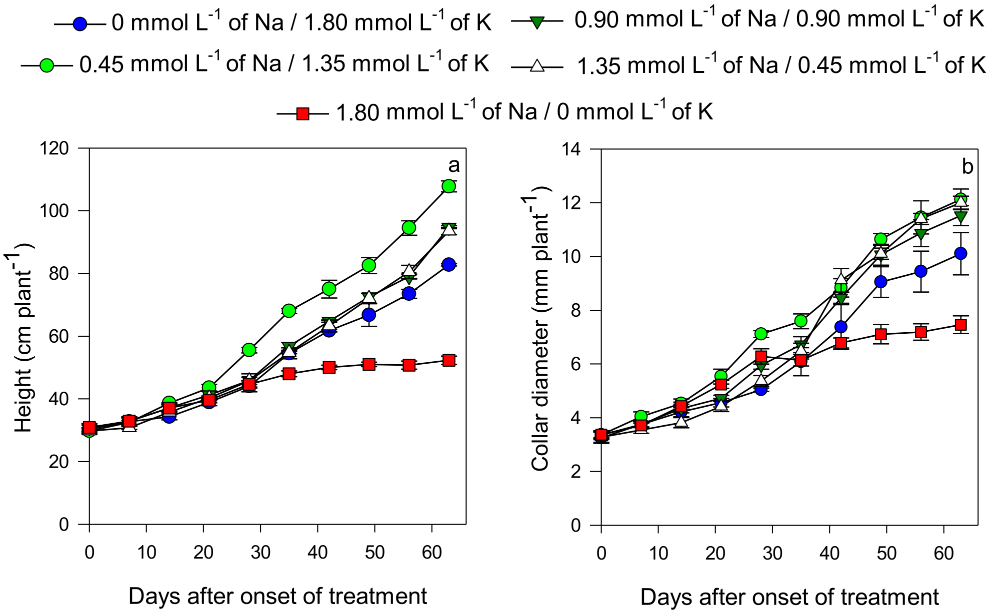

Fig. 2. Weekly growth in height (a) and collar diameter (b) of *Eucalyptus* seedlings subjected to K replacement by Na in the nutrient solution. Vertical bars indicate standard errors between blocks (n = 4).

Table 1
Root, shoot, total dry matter yield and root: shoot ratio of *Eucalyptus* seedlings subjected to K replacement by Na in the nutrient solution.

Treatments	Dry Matter Production (g plant^{-1})*			
	Root	Shoot	Total	Root: Shoot Ratio
0	13.1 ± 2.6a	56.7 ± 10.5a	69.8 ± 13.1a	0.23 ± 0.01b
0.45	9.6 ± 1.4b	53.6 ± 1. 8a	63.2 ± 3.0a	0.17 ± 0.02c
0.9	9.4 ± 1.1b	52.1 ± 3.5a	61.6 ± 4.4a	0.18 ± 0.01c
1.35	7.0 ± 2.5c	36.2 ± 9.9b	43.2 ± 12.4b	0.19 ± 0.01c
1.8	3.6 ± 0.5d	13.3 ± 1.4c	16.9 ± 1.9c	0.27 ± 0.01a
Models	y = 12.78–4.76***Na	y = 54.57 + 12.91^{ns}Na-19.78***Na^{2}	y = 66.75 + 10.18^{ns}Na-20.82**Na^{2}	y = 0.22–0.14**Na + 0.09***Na^{2}
R^{2}	0.67	0.89	0.87	0.65

Data represent mean values and standard errors between blocks (n = 4).*Influenced statistically (by F test $p < 0.05$) by Na rates. ***, ** and ^{ns} represents significance at 0.1%, 1% and not significant at 5% by F test ($p < 0.05$), respectively. Different letters indicate significant differences according to Tukey test ($p < 0.05$).

corresponding, respectively, to a decrease of 45%, 49% and 38% in relation to the control treatment (Na rate of 0 mM). The Na application was not significant in the C_i/C_a ratio, showing an average value of 0.81 (Fig. 4d). Besides, WUE values were significantly changed with increased Na concentration in the nutrient solution (Fig. 4e), with higher WUE observed in plants grown under Na rates of 0.90 mM. Increasing Na concentration from 0 to 0.90 mM in the solution augmented WUE of *Eucalyptus* seedlings by 46% (Fig. 4e).

3.4. Leaf water potential (Ψ_w), $\delta^{13}C$ and the correlation among leaf $\delta^{13}C$, shoot dry matter yield, A, WUE and g_s

Predawn Ψ_w values were not affected by Na, with a mean value of −0.31 Mpa. At noon, a significant increase (less negative) in Ψ_w with increasing Na application was observed (Fig. 5a). At noon, plants with intermediary rates of Na showed lower Ψ_w, whereas the highest Ψ_w was found in the higher rate of Na. Na supply had a significant influence (Fig. 5b) on leaf carbon isotopic composition (δ^{13}C ‰) of *Eucalyptus* seedlings. Carbon isotope signatures of *Eucalyptus* seedling leaves reflected imposed K nutritional imbalances, notably in plants under higher Na concentration in the solution. Increasing Na concentration in the nutrient solution up to 0.9 mM decreased leaf ^{13}C composition values (−32.4‰). On the other hand, Na supply of 1.8 mM providing the highest value of δ^{13}C (−31.2‰). In addition, these significant differences in δ^{13}C between treatments (Fig. 5b) were translated to substantial variation in WUE, corresponding to a decrease of 43% in WUE with enrichment of δ^{13}C in plants leaves (Fig. 5c). Overall, we observed a closed and negative correlation among δ^{13}C, WUE, g_s (Fig. 5c), and among δ^{13}C, shoot dry matter and *A*. (Fig. 5d).

3.5. Leaf area, specific leaf area, stomatal density and stomatal number

Sodium supply affected significantly leaf area (LA) and specific leaf area (SLA) (Table 2). The absence of Na in the nutrient solution favored the highest LA (0.87 m^{2} plant^{-1}). On the other hand, LA and SLA decreased considerably in the higher Na rate (1.80 mM), with a reduction of 88% and 54%, respectively, compared to control treatment (0 mM of Na). Increased Na concentration in the solution had no significant effect on stomatal density (SD), that showed mean value of 556.5 stomates mm^{-2}. Despite this, intermediary rates provided trend to higher SD. However, there was a significant effect of Na application on the total number of stomata per leaf (Table 2).

4. Discussion

4.1. Eucalyptus seedlings growth and dry matter yield

A small Na application rate in nutrient solution, in combination with K, was beneficial to the growth of the *Eucalyptus* seedlings, which was observed for the majority of variables analyzed in the current investigation, such as plant biomass yield, plant growth (height and collar diameter), leaf gas exchange and leaf carbon isotope discrimination (δ^{13}C ‰). Under the growth conditions assayed in this investigation, increased Na availability in the nutrient solution led to substantial decreases in stomatal conductance (g_s), CO_2 assimilation rate (*A*), transpiration rate (*E*), enriched δ^{13}C in leaf tissues measured and corresponding changes in plants WUE. In this sense, Almeida et al. (2010) also observed a positive effect of Na supply on height and biomass of *E. grandis* plants, on which it were growing in soil with low K available. Likewise, a positive growth response of *E. camaldulensis* seedlings to minimal use of NaCl was also observed by Rawat and Banerjee (1998). However, the remaining question is how much K-replacement by Na can be done before affecting negatively the physiology, development and growth of young *Eucalyptus* plants. In this study, the reduction in plant growth occurred in plants exposed to 1.80 mM of Na (exclusive Na supply). This effect was likely caused by nutritional imbalance due to K deficiency (Marschner, 2012), as visual symptoms of K deficiency were clearly observed (Fig. 1), showing premature foliar senescence, chlorosis followed by marginal necrosis in leaves and by root malformation.

The total dry matter yield of plants (Table 1) subjected to Na concentration in the nutrient solution of 0, 0.45 and 0.90 mM were about four times higher than in those plants grown under the highest Na rate (1.8 mM of Na), which means that just a smaller proportion of K can be replaced without decreasing shoot and total plants biomass. Adams et al. (2005) pointed out that the lowest NaCl application (0.1 mM) promoted higher total dry matter yield, notably in *E. spathulata* plants. Conversely, as expected, the highest concentrations of NaCl (200 and 300 mM) reduced growth of *Eucalyptus ravertiana*, *E. spathulata, E. sargentii* and *E. loxophleba* relative to that recorded at the lowest concentration – Na supply of 0.1 mM (Adams et al., 2005). According to Tester and Davenport (2003), Na^{+} cannot replace all the 50 enzymes activated by K^{+}, which indicates that the fully K-replacement by Na is prejudicial for *Eucalyptus* seedlings, as observed in growth and dry matter production in the higher rate (1.8 mM of Na). The stunted growth (Fig. 2), dry matter yield (Table 1) and yellowing in leaf margins (Fig. 1b) we observed at 1.8 mM of Na are often associated with K deficiency (Wang et al., 2013). The root: shoot ratio values were a response to the wide reduction in shoot dry matter production with higher Na rates, which means that the shoot reduced proportionally more than the root system. The partial replacement of K by Na is known to enhance plant water balance, and to maintain cell turgor and cell expansion (Wakeel et al., 2011), and thus promote plant growth (Table 1). According to Wakeel et al. (2011), since Na is cheaper than K fertilizers, partial K substitution by Na can represent a great reduction in cost production. Thus, the estimated optimal application rate of

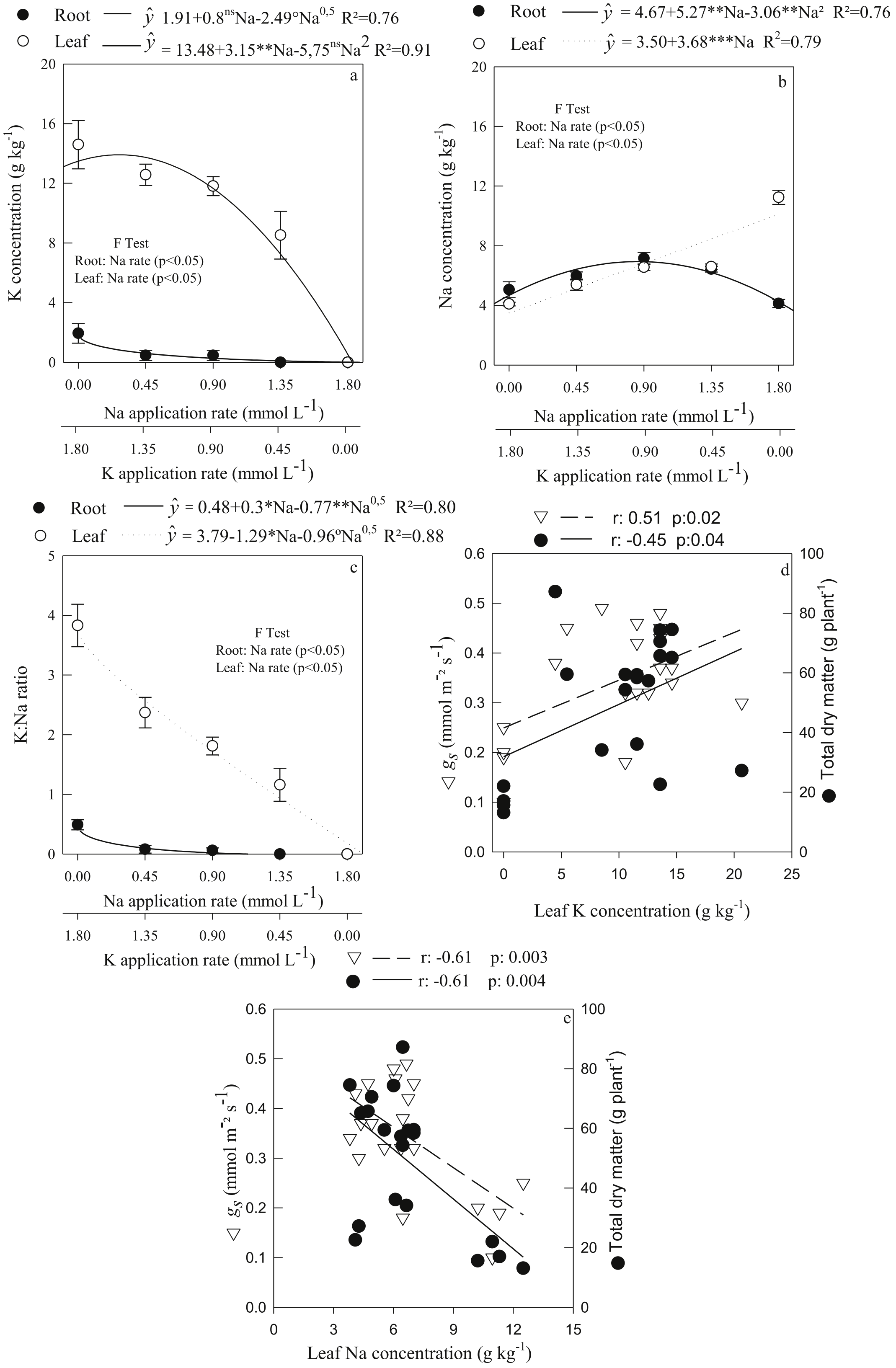

Fig. 3. Leaf K- (a) and leaf Na-concentrations (b) leaf K:Na ratio (c), and relationships among leaf K- (c) and Na- (d) concentrations and g_s or total dry matter in the root and leaf of *Eucalyptus* seedlings subjected to K replacement by Na in the nutrient solution. ***, **, *, ° and ^{ns} represents significance at 0.1%, 1%, 5% and 10% and no significance at 5% by the F test ($p < 0.05$), respectively. Vertical bars indicate standard errors between blocks (n = 4).

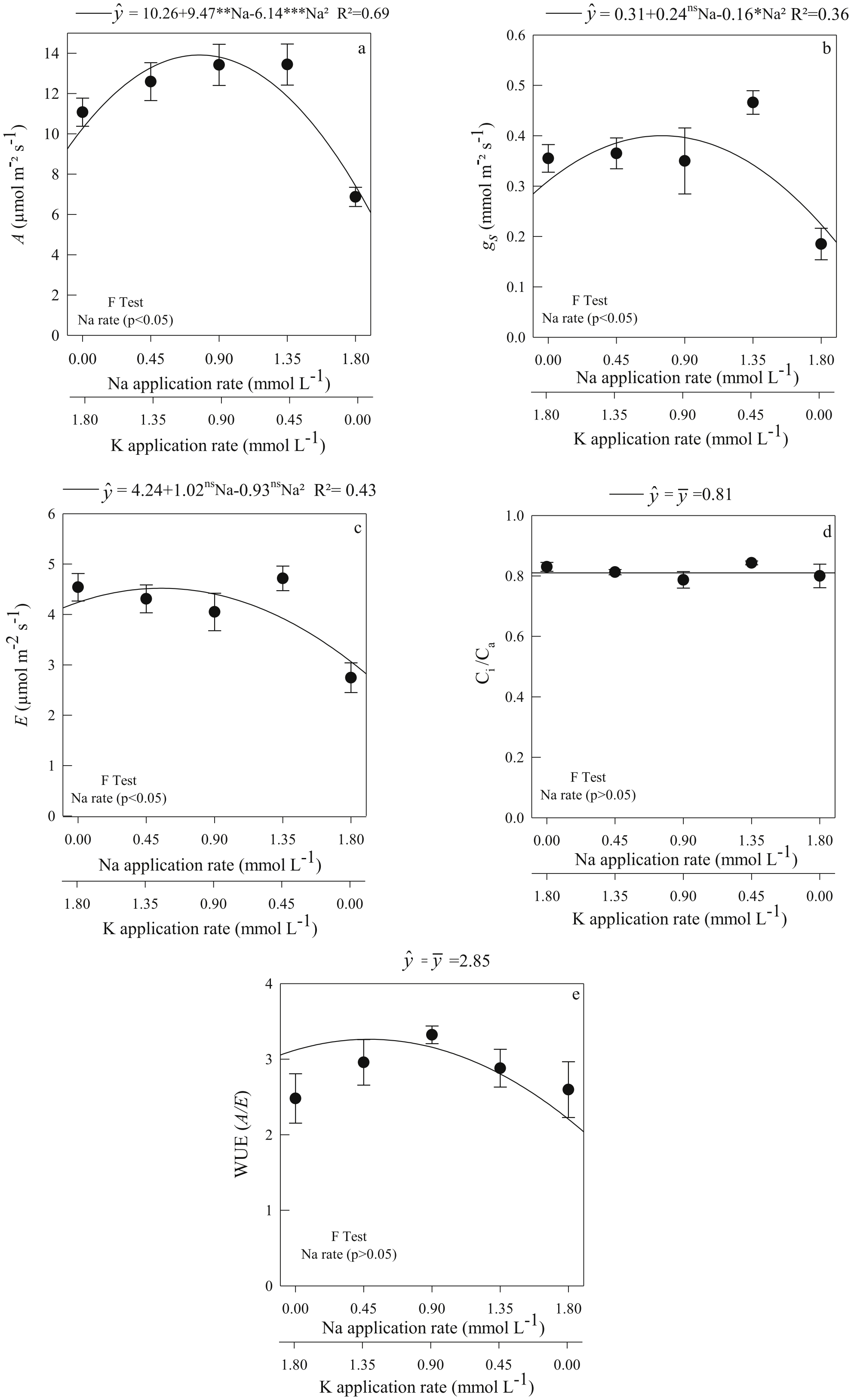

Fig. 4. Leaf gas exchange values of *Eucalyptus* seedlings subjected to K replacement by Na in the nutrient solution: photosynthesis- *A* (a), stomatal conductance-g_s (b), transpiration-*E* (c), leaf-internal (C_i) and external (C_a) CO_2 concentration ratio- Ci/Ca (d) and water use efficiency-WUE (e). ***, **, *, ° and ^{ns} represents significance at 0.1%, 1%, 5% and 10% and no significance at 5% by F test ($p < 0.05$), respectively. Vertical bars indicate standard errors between blocks (n = 4).

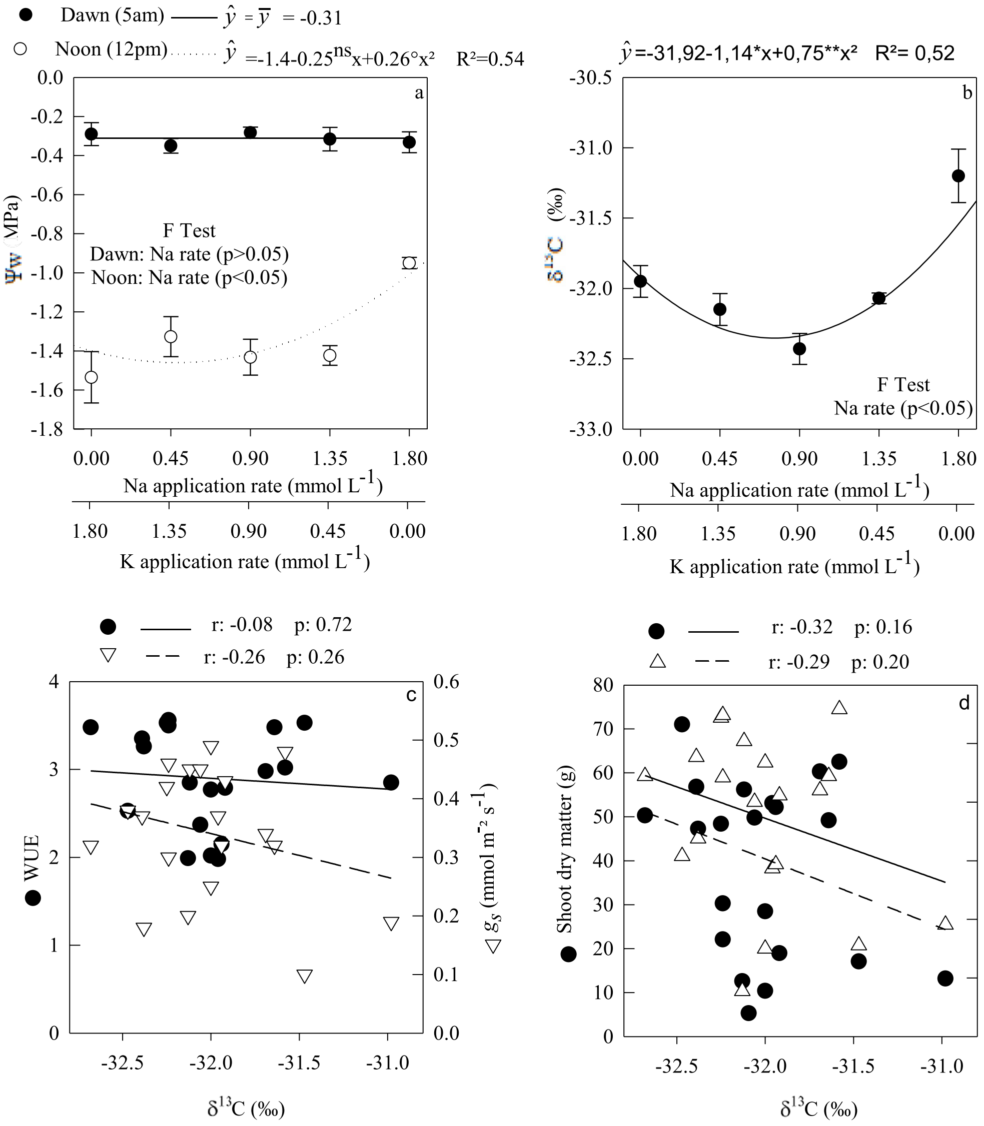

Fig. 5. Leaf carbon isotope composition $\delta^{13}C$ ‰ (a), leaf water potential-Ψ_w (b) and the correlation among $\delta^{13}C$, WUE and g_s (c) and among $\delta^{13}C$, shoot dry matter and A (d) of *Eucalyptus* seedlings subjected to K replacement by Na in the nutrient solution. **, *, $^{\circ}$ and ^{ns} represents significance at 1%, 5%, 10% and not significance at 5% by F test ($p < 0.05$), respectively. Vertical bars indicate standard errors between blocks (n = 4).

0.25 mM of Na means 14% of substitution and could bring a greater reduction in cost to *Eucalyptus* plantation management without a decrease in its yield.

4.2. Plant tissues K- and Na-concentration

The leaf K-concentration in adult *Eucalyptus* plantations varies from 5.5 to 8.5 g kg^{-1} (Gonçalves, 2010). Besides, the nutritional efficiency among species of *Eucalyptus,* clones, and plant ages does not follow

Table 2

Leaf area-LA, specific leaf area-SLA, stomatal density-SD (stomates mm^{-2}) and number of stomates per leaf of *Eucalyptus* seedlings subjected to K replacement by Na in the nutrient solution.

Treatments	Leaf area (m^2 plant^{-1})*	Specific leaf area (m^2 kg^{-1})*	Stomatal density (stomates mm^{-2})	Stomates (10^{7}plant^{-1})*
0	0.87 ± 0.16a	28.5 ± 0.9b	569.9 ± 7.5 ab	49.9 ± 5.77a
0.45	0.76 ± 0.01 ab	29.8 ± 0.7b	581.4 ± 14.0a	44.3 ± 1.41a
0.9	0.78 ± 0.02b	29.8 ± 0.9b	586.2 ± 12.0a	46.1 ± 2.12a
1.35	0.61 ± 0.09c	33.1 ± 2.7a	562.1 ± 13.1 ab	33.9 ± 3.02b
1.8	0.10 ± 0.01d	12.9 ± 1.2c	469.1 ± 3.2b	5.3 ± 0.91c
Models	$y = 0.79 + 0.29^{\circ}Na - 0.36^{***}Na^2$	$y = 25.77 + 19.91^{**}Na - 14.37^{***}Na^2$	556.5	$y = 45.57 + 18.26^{\circ}Na - 21.92^{***}Na^2$
R^2	0.91	0.75	0.64	0.90

Data represent mean values and standard errors between blocks (n = 4).*Influenced statistically (by F test $p < 0.05$) by Na rates. ***, ** and $^{\circ}$ represents significance at 0.1%, 1% and 10% by F test ($p < 0.05$), respectively. Vertical bars indicate standard errors between blocks (n = 4). Different letters indicate significant differences according to Tukey test ($p < 0.05$).

equally patterns (Santana et al., 2002; Pinto et al., 2011). In the field, Almeida et al. (2010) observed a foliar Na concentration of approximately 1.0 g kg^{-1} on treatments with 68 kg ha^{-1} of Na as NaCl in 1 year E *grandis*. Genotype, period of cultivation and plant age might explain the great difference for foliar Na concentrations among studies. Battie-Laclau and Laclau (2014) observed a leaf K:Na ratio about 3.5 in *E. grandis* in a field experiment with 0.45 mol m^{-2} of Na as NaCl, 103 days after leaf emergence. Decreased leaf K concentration due to concomitant decreasing K and increasing Na rates in the nutrient solution, indicates that there is an optimal K:Na ratio in the nutrient solution, as well as in the plant tissue. The leaf K concentration positively correlated with g_s and total dry matter (Fig. 2d), since increase in leaf K concentration provides greater Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) activity and, consequently, higher photosynthetic rate (Lu et al., 2016). In this study a negative correlation was observed with Na supply, since plants with higher Na leaf concentration showed decreased g_s and total dry matter production (Fig. 2e).

4.3. Leaf gas exchange

Changes in g_s relate to the control of water loss and CO_2 assimilation, simultaneous processes as observed by Battie-Laclau and Delgado-Rojas (2016) in *Eucalyptus* plants. The small K replacement by Na altered g_s, increased A and might have mitigated anatomical and biochemical damages caused by K deficiency, as also observed in *E. grandis* plantations (Battie-Laclau and Laclau, 2014). Besides the higher A, the C_i/C_a ratio values did not change by Na application, as also observed in *Eucalyptus* clones with boron supply by Mattiello et al. (2009) and N supply by Ferreira et al. (2015), which leaf-internal CO_2 concentration was stable and not limiting for A, leading to a small variation on the other variables evaluated.

The higher WUE was observed at intermediary Na rates as 0.45 and 0.90 mM, similarly to A, whose increase was not accompanied by E (transpiration rate) thus, promoting higher carbon assimilation with less water transpired, leading WUE to higher values (around 3). Those plants that had higher WUE (Fig. 4e), also showed greater height and collar diameter (Fig. 2a and b) and total dry matter production (Table 1), indicating that productive plants lose more water by transpiration rate, and then produce more wood per water used, as observed in *Eucalyptus* spp. by Mattiello et al. (2009), Forrester et al. (2012) and Otto et al. (2014).

The leaf K:Na ratio of 3.41 (Fig. 3c), provided by the estimated Na rate of 0.25 mM (1.55 mM of K) was correlated to the higher total dry matter production (Table 1). With this, it can be inferred that this optimal ratio provided a better gas exchange condition in *Eucalyptus* seedlings, leading plants to produce a higher yield. Otherwise, the rate 1.80 mM of Na provided the lowest values for A, g_s and E, as well as lower growth (Fig. 2), lower total dry matter (Table 1) and higher leaf Na concentration (Fig. 3b), leading to leaf K-deficiency symptoms (Fig. 1).

4.4. Leaf water potential (Ψ_w) and $\delta^{13}C$ and its correlation with shoot dry matter yield, A, WUE and g_s

K and Na applications were beneficial by enhancing cell turgor in fully expanded *E. grandis* leaves, which improved osmotic adjustment through a reduction in osmotic potential (Adams et al., 2005; Battie-Laclau and Laclau, 2013; 2014), stimulating cell division and elongation rates (Pantin et al., 2011). As seen in this study at noon (Fig. 5a), the rate of 1.8 mM of Na promoted the highest Ψ_w, indicating that high Na concentrations can cause stomata deregulation, as showed by the huge decrease in A, g_s and E (Fig. 4a, b and 4c).

In water stress condition, stomata closure is a mechanism to reduce water loss and consequently affects specific leaf area (SLA) and A (Schulze et al., 2006), and can be measured by $\delta^{13}C$, the isotopic ratio of ^{13}C–^{12}C (Farquhar et al., 1982). There is a negative correlation between $\delta^{13}C$ and growth in *Eucalyptus* (Osório and Pereira, 1994; Le Roux et al., 1996; Ngugi et al., 2003) and this is as a result of plant SLA changes, leading to gas exchange reduction and stomata closure (Osório and Pereira, 1994). It can be observed that higher A and g_s lead consequently, to better WUE (Farquhar et al., 1989) and less negative $\delta^{13}C$ values, thus, plants fixed more ^{12}C than ^{13}C, which was expected, since ^{13}C diffusion is slower than ^{12}C (Farquhar et al., 1982). The K deficiency (0 mM of K) (Fig. 5b) leads to a consistent increase in $\delta^{13}C$ values in *Eucalyptus* plants. Thus, $\delta^{13}C$ values can be used as an indicator for ranking WUE, allowing select appropriated clones and improved yield under drought conditions (Le Roux et al., 1996).

Despite we have detected significant differences in $\delta^{13}C$ among treatments (Fig. 5b), this did not translate to substantial variation in WUE (Fig. 5c). Although not significant, there was a trend for negative correlation among $\delta^{13}C$ and WUE, g_s, shoot dry matter and A (Fig. 5c and d), as also observed by Farquhar et al. (1989), Le Roux et al. (1996), Zhu et al. (2016) and Mårtensson et al. (2017). Probably, under conditions evaluated in this study, differences among the treatments with Na supply, carbon isotope discriminations were not large enough to translate to differences in plants WUE or that how many times the gas exchange measurements obtained here were not enough to better describe the real time-integrated changes in WUE (see, Farquhar et al., 1982; Farquhar, 1983). It is important to bear in mind that measuring WUE from leaf gas exchange and predicting from $\delta^{13}C$ reflects different time scales of assimilated CO_2, where measurements using gas exchange are instantaneous while carbon isotope discrimination involves integration of the measured plant growth over a time course of the experiment (Smith et al., 2018). Overall, this inconsistent result related to the use of instantaneous WUE (A/E) rather than using the season-long WUE can be explained to the fact of measurements of accumulated shoot biomass and water consumption (up take, transpiration rate and evaporation) over the entire experimental period (see Battie-Laclau and Delgado-Rojas, 2016; Bond and Stock, 1990; Jordan-Meille et al., 2018; Tavakol et al., 2018).

4.5. Leaf area, specific leaf area, stomatal density and stomatal number

The size and number of leaves determined the photosynthetic surface area, being an important plant attribute from both physiological and economical perspectives (Battie-Laclau and Laclau, 2013). According to Battie-Laclau and Laclau (2013), LA increased 31% in Na supplied trees, compared to the control treatment, by enhancing leaf size, number, turgor longevity and the mean area of individual leaves, otherwise, still was significantly lower than in K supplied adult trees (Epron and Laclau, 2012). The lower LA and SLA in exclusive Na supply (1.8 mM of Na) can be partially explained by early senescence leaves in Na-induced K deficiency conditions, as was also observed by Marschner (2012).

According to England and Attiwill (2011), SD and its morphology can influence g_s and consequently, regulate A and E. Smaller SD can increase the size of the stomata and chloroplast concentration in guard cells, and consequently, increase the WUE (Doheny-Adams et al., 2012). The trend to higher SD in intermediary Na rates was not harmful, as observed by Doheny-Adams et al. (2012), since these plants had higher total dry matter production and LA (Tables 1 and 2, respectively).

Thus, it was observed that intermediary rates of Na application provided higher LA and SLA (Table 2), which promoted higher photosynthetic area, as expected, since greater leaf surface provides higher A and E (Wright et al., 2006), resulting in greater growth (Fig. 2) and total dry matter (Table 1), as well as higher WUE (Fig. 4e) and lower $\delta^{13}C$ in the *Eucalyptus* clone seedlings. Such contestation of the beneficial effects of Na application in smaller rates, in addition to K supply in *Eucalyptus* seedlings in nutrient solution, indicates the possibility of the use of Na in culture fertilization, reducing the cost of K supply with an alternative source (i.e., NaCl).

4.6. Final considerations, and outlook

Overall, although woody plant species are different from annual crops and model plant (*e.g.*, *Arabidopsis thaliana*, or *Setaria viridis*), we can catch a glimpse into the potential of plant molecular approaches supported by the new “omics” tools in order to understand mechanisms of K^+ and Na^+ uptake, transport, ion replacement and its utilization in *Eucalyptus* trees, as well as to understand how water use efficiency and plant growth are shaped in function of spatio-temporal dynamics of water and nutrient availability in the soils. Likewise, as the *Eucalyptus* genome assembly was published a few year ago, some related investigations may look into the relevant gene expressions for K^+ and Na^+ uptake, transport and its utilization - *e.g.*, selective ion channels such as AKT1 (inward rectifying K^+ channel), HKT (high-affinity K^+ channels), non-selective ion channels (NSCC), and outward rectifying K^+ channels (ORC) - in root and shoot of *Eucalyptus* plants with contrasting K nutritional efficiency, and growing under different K and Na availability. However, this hypothesis has not yet been investigated.

5. Conclusions

Eucalyptus plants showed a positive response to small K replacement by Na in nutrient solution, since this element affected directly the plants' physiology, improved growth, dry matter production, gas exchange (A, E, g_s and WUE), LA, SD, and guided to a lower values of leaf carbon isotope ($\delta^{13}C$ ‰). The estimated Na application rate (0.25 mM) lead *Eucalyptus* seedlings to the higher total dry matter production, with a leaf K:Na ratio of 3.41, which means 14% substitution of K by Na. However, in the highest Na rate (1.8 mM) the Na-induced K deficiency reduced plant growth and physiology, which deregulated their osmotic adjustments. Collectively, our results conclude that substitution of 25% of K by Na (0.45 mM of Na) provided significant gains in nutritional status and positive plant physiological responses by increasing WUE, stomatal diffusion, and by augmenting CO_2 uptake efficiency. This nutritional management can therefore be an alternative option to optimize yields and resource use efficiencies in *Eucalyptus* cultivation.

Author contributions

NSM, EVOF and JLMG designed and performed this research. NSM, EVOF, JLMG, JCAJ and JL analyzed the data. JCD, LJM, and JL revised this draft by rewriting, discussion and commenting. All authors contributed in the same way to the preparation of all the parts of this manuscript.

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