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Fertilization increases the functional specialization of fine roots in deep soil layers for young *Eucalyptus grandis* trees☆

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ABSTRACT

Functional specialization of fine roots was found for *Eucalyptus grandis* trees at harvesting age (6 years) on tropical soils. Aiming to elucidate whether functional specialization is a ubiquitous feature of eucalypts, we focused on its changes with ontogeny, tree nutrient status and soil depth. We studied the potential uptake of N, K and Ca by 2-year-old *E. grandis* trees, as a function of soil depth and NPK fertilization. We injected $NO^{-}{}_{3}$-^{15}N, Rb^{+} (K^{+} analogue) and Sr^{2+} (Ca^{2+} analogue) tracers simultaneously in a solution at depths of 10, 50, 150 and 300 cm in a sandy Ferralsol soil. A complete randomized block design was set up with three replicates of paired trees per injection depth, in fertilized and non-fertilized plots. Recently expanded leaves were sampled at 70 days after tracer injection. Determination of foliar Rb, Sr concentrations and x(^{15}N) allowed estimating the relative uptake potential (RUP) and the specific RUP (SRUP), defined as the ratio between RUP and fine root length density (RLD) in the corresponding soil layer. Various root traits were measured at each depth. Foliar N and K concentrations were higher in fertilized than in non-fertilized trees. The RUP of NO_3^--^{15}N decreased sharply with soil depth and the highest values of the SRUP of NO_3^--^{15}N were found at a depth of 50 cm. The RUP of Rb^+ and Sr^{2+} did not change with soil depth, whilst the SRUP of Rb^+ and Sr^{2+} were higher at the depth of 300 cm than in the topsoil, concomitant with an increase in root diameter and a decrease in root tissue density with depth. The SRUP of Rb^+ and Sr^{2+} at a depth of 300 cm were on average 136 and 61% higher for fertilized trees than for non-fertilized trees, respectively. Fine roots of young *E. grandis* trees showed contrasting potential uptake rates with soil depth depending on the nutrient. Fertilization increased the uptake rate of Rb^+ and Sr^{2+} by unit of root length in deep soil layers. Functional specialization of fine roots for cations of low mobility depending on depth previously shown at harvesting age also occurs in young *E. grandis* plantations and increases with fertilization application. This mechanism helps explaining very low amounts of cations lost by leaching in *Eucalyptus* plantations established in deep tropical soils, even in highly fertilized stands.

1. Introduction

Forest plantations accounted in 2015 for some 291 million hectares (7% of the world forest areas), (FAO, 2015) and play an increasing role to satisfy the increase in global wood demand (Keenan et al., 2015; Paquette and Messier, 2010). Eucalypt plantations cover about 20 million hectares around the world and are expanding rapidly in tropical and subtropical regions to provide raw material for wood, paper, and biofuel products as well as large amounts of firewood and charcoal for domestic uses (Booth, 2013). *Eucalyptus grandis* Hill ex Maiden, is one of the most planted *Eucalyptus* species owing to its high productivity (Stape et al., 2010), and adaptation to various environments (Binkley et al., 2017; Costa et al., 2017).

High productivity of commercial eucalypt plantations in Brazil

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largely depends on nitrogen (N), phosphorus (P) and potassium (K) applications (Gonçalves et al., 2013). However, the potential negative environmental impacts of fertilizers might limit their use in eucalypt plantations in the future (Brunelle et al., 2015). Even though fertilizers are applied in the topsoil, significant amounts of K and N are leached and taken up by *Eucalyptus* trees down to a depth of 3 m (Laclau et al., 2010). Fine roots, usually defined according to a diameter-based cut-off ≤2 mm (Freschet et al., 2017) play a major role in the capture and transport of water and nutrients needed for plant growth (Pregitzer et al., 2002; McCormack et al., 2015; Fort et al., 2017). Although tree fine roots are usually more abundant in the shallow soil layers than in depth (Brassard et al., 2009; Laclau et al., 2013; Pinheiro et al., 2016), their presence was observed in very deep soil layers in tropical or subtropical fast-growing plantations and natural forests (Nepstad et al., 1994; Christina et al., 2017; Germon et al., 2017; Fan et al., 2017), e.g. 17 m deep in 3.5 year-old *Eucalyptus* plantation in Brazil (Christina et al., 2011). The physiological function of deep fine roots is still questioned (Al Afas et al., 2008; Maeght et al., 2013). Deep-rooting is an important strategy to increase the amount of water available for the trees, and to cope with seasonal droughts (Maeght et al., 2013; Christina et al., 2017; Broedel et al., 2017; Fan et al., 2017). However, the processes that control the uptake of ions such as NO_3^-, K^+ or Ca^{2+} in very deep soil layers are still poorly understood in forest ecosystems (Iversen, 2010; Hinsinger et al., 2011; Binkley, 2015). Such information would be useful to assess the need to apply fertilization at various dates after planting (Laclau et al., 2010).

Various factors drive nutrient uptake by tree roots, including soil nutrient availability, ion mobility in soil solutions, root traits or root ion transporters (Chapman et al., 2012; Costa et al., 2017; Kulmatiski et al., 2017). Contrasting potential uptake rates with depth depending on the nutrient were found for *Quercus robur L., Fagus sylvatica L. and Picea abies* (L.) Karst. in Northern Europe (Göransson et al., 2007, 2008) and for *E. grandis* in Brazil (da Silva et al., 2011). Fine roots of mature *E. grandis* trees exhibited, by unit of root length, greater uptake capacity of Rb^+ (analogue of K^+) and Sr^{2+} (analogue of Ca^{2+}) at a depth of 3 m than in the topsoil (da Silva et al., 2011). By contrast, a specialization of upper fine roots was likely in NO_3^- uptake. These results found at harvesting age were consistent in dry and wet seasons, for clayey and sandy soils. However, no information is available for young trees and the consequences of tree nutrient status on the functional specialization of fine roots are still unknown. Root specialization might be explained by many factors, as changes in specific transporter activity and mass flow rates from the soil to the roots through changes in root hydraulic conductivity (Costa et al., 2017). High-affinity transporters in plant, such as K and N-transporters, can be activated at very low nutrient concentrations allowing efficient nutrient uptake (Schachtman and Schroeder, 1994; Kiba and Krapp, 2016). On the opposite, Bao et al. (2011) showed that AtNRT2.1, an inducible high-affinity NO_3^- transporter in *Arabidpsis thaliana* was upregulated by phosphate and sulphate supply. Higher nutrient availability can increase tree nutrient uptake as shown for *E. grandis* (Rowe et al., 2008; Costa et al., 2017).

Moreover, to the best of our knowledge, no specific root functional traits have been associated with the functional specialization of *Eucalyptus* fine roots along the soil profile. Water and nutrient availabilities are highly dependent on soil depth in tropical eucalypt plantations (Mareschal et al., 2013; Versini et al., 2014). This heterogeneity throughout the soil profile leads to contrasting root functional traits that are highly sensitive to heterogeneous resource distributions (Ostonen et al., 2007). Prieto et al. (2015) measured root functional traits in 20 plant communities located in 3 climatic zones (tropical, Mediterranean and montane) along a land-use gradient. Fine roots exhibited different suites of functional traits (e.g. root diameter or Specific Root Length, SRL) at different soil depths suggesting a difference in root function and foraging capacity. Roots with higher SRL were found in less fertile soils. Pate et al. (1995) showed for Australian species that deep roots had conductivities up to 15 times higher than roots of similar diameter in the topsoil layers, which could help to explain the ability of *Eucalyptus* trees to use efficiently the resources in deep soil layers despite very low fine root densities.

The study set out to assess the potential uptake of N, K and Ca by 20-month-old *E. grandis* trees as a function of soil depth and fertilization at planting. We used NO_3^--^{15}N, Rb^+ and Sr^{2+} tracers simultaneously injected close to trees, at several depths down to 3 m, in a completely randomized experiment including NPK-fertilized and control (non-fertilized) plots. We hypothesized: (i) a functional specialization of fine roots for young eucalypt trees with higher potential uptake rate (per unit of fine root length) for NO_3^--^{15}N in the surface soil and for Rb^+ and Sr^{2+} in the deep soil layers, as shown in mature eucalypt plantations, and (ii) an increase in functional specialization of fine roots when tree nutrient status is improved by fertilization.

2. Materials and methods

2.1. Study site

The study was carried out in the Itatinga experimental station of São Paulo University (23°02′S, 48°38′W), at 860 m above mean sea level. Over the 15 years prior to planting, the mean annual rainfall was 1360 mm, with a dry and cold season from June to September. The soils are very deep Ferralsols according to FAO classification (FAO, 2014) developed on Cretaceous sandstone with a water table at a depth of approximatively 17 m (Christina et al., 2011). Soil chemical analyses down to a depth of 3 m are given in Table 1.

2.2. Experimental design

The experiment was conducted in a complete randomized block design with three blocks and two treatments. Seedlings were planted at a density of 1111 trees ha^{-1} (3 m × 3 m spacing) on May 2014. Each plot had a total area of 48 m × 48 m and an inner plot of 36 m × 36 m with two buffer rows. Within each block, there were plots of *E. grandis* without (F−) or with fertilization (F+) applied at planting: 125 kg ha^{-1} P, 121 kg ha^{-1} N, 136 kg ha^{-1} K, 45 kg ha^{-1} B and 30 kg ha^{-1} FTE (*Fritted Trace Element*, micronutrients). Factorial fertilization trials at the study site showed that the amounts of nutrients applied were non-limiting for *Eucalyptus* tree growth (Laclau et al., 2009). Fertilizers were dug into the soil below each tree. Higher N and K concentrations were found in leaves of fertilized than non-fertilized trees (Table S1). The study was carried out on January 2016, at 20 months of age during the rainy period. The tracers were applied at four depths (i.e. 10, 50, 150 and 300 cm) in each block, in both F+ and F− treatments. For each depth, NO_3^--^{15}N, Rb^+ and Sr^{2+} tracers were injected together into seven holes around two neighbouring *Eucalyptus* trees with the same basal area as the average of the stand (da Silva et al., 2011). The position of the holes was ¼ (0.75 m) and ½ (1.5 m) of the inter row on both sides of the planting row, at mid-distance (1.5 m) from the two sampled trees in the planting row and at mid-distance (1.5 m) from the two nearest neighbours in the planting row (Fig. 1). The pairs of trees were located more than 16 m apart to prevent root competition for nutrient uptake. Gravimetric water contents (around 10%) were not significantly different between treatments regardless of the depths.

2.3. Tracer application

A labelled solution was prepared in the laboratory, one day before application. RbCl (100 g), $SrCl_2$ (215 g) and NH_4-$^{15}NO_3$ (10 atom% NO_3-^{15}N) (652 g) were dissolved in 3360 mL of distilled water. The solution was maintained at a temperature of 4 °C until application in the field. Holes were drilled down to the target application depth using a 35-mm diameter stainless steel auger. A 25-mm diameter PVC tube was inserted into each hole to avoid contamination of upper soil layers

Table 1
Main physical and chemical soil attributes before fertilization at the experimental site.

Depth	Clay	Silt	Sand	N	C	pH $CaCl_2$	P	H + Al	K	Ca	Mg	SB	CEC
(cm)	%						$mg\,kg^{-1}$	$mmol_c\,kg^{-1}$					
0–20	23.4 ± 2.2	5.75 ± 0.2	70.81 ± 2.3	0.037 ± 0.002	0.58 ± 0.09	3.73 ± 0,04	4.30 ± 0.45	83.82 ± 8.09	0.43 ± 0.04	1.02 ± 0.10	0.65 ± 0.03	2.10 ± 0.1	102.19 ± 10.24
40–60	23.98 ± 2.4	3.88 ± 0.5	72.13 ± 2.8	0.025 ± 0.002	0.39 ± 0.04	3.88 ± 0.03	3.05 ± 0.35	48.65 ± 2.6	0.28 ± 0.02	0.88 ± 0.05	0.62 ± 0.03	1.78 ± 0.06	50.44 ± 2.69
100–200	29.02 ± 2.7	4.42 ± 0.3	66.57 ± 3.0	0.016 ± 0.0002	0.23 ± 0.04	4.04 ± 0.03	2.07 ± 0.26	30.64 ± 1.8	0.19 ± 0.02	0.77 ± 0.04	0.62 ± 0.03	1.58 ± 0.06	32.22 ± 1.84
200–300	31.08 ± 2.3	5.02 ± 0.4	63.95 ± 2.4	0.009 ± 0.00	0.13 ± 0.02	4.19 ± 0.03	2.15 ± 0.28	24.64 ± 2.1	0.18 ± 0.02	0.80 ± 0.06	0.62 ± 0.03	1.60 ± 0.07	26.24 ± 2.14

Total N and C contents determined using a CHN analyzer. P was determined through resin extraction and colorimetry; K was determined by Mehlich-1 and spectrophotometry; Ca and Mg were determined by KCl extraction and atomic absorption; SB, sum of bases; CEC, cation exchange capacity. The standard errors are given (n = 6 for C, N, clay, silt and sand contents and n = 24 for the other elements).

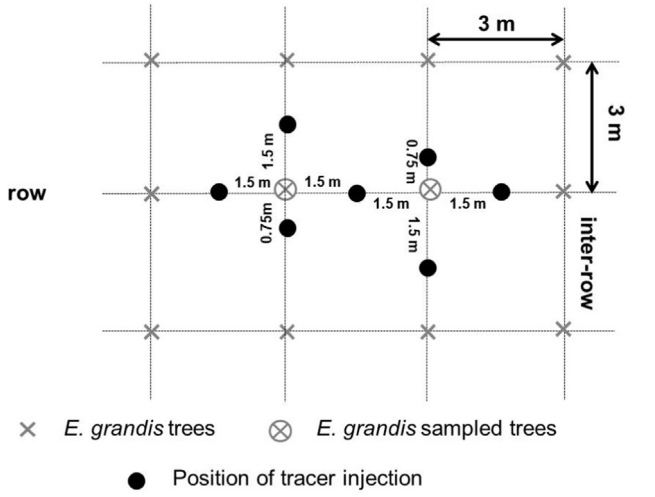

Fig. 1. Layout of the positions where tracers were injected for each depth and each pair of sampled trees. Leaf samples were collected from the same pair of trees (adapted from da Silva et al., 2011).

during tracer application. A 4-mm diameter polyethylene tube, attached to an iron rod, was inserted into the PVC tube and 20 mL of the labelled solution was injected at the selected depth. Then, 10 ml of distilled water was injected to rinse the syringe and the polyethylene tube. At each position we injected 0.42 g of Rb^+; 0.42 g of Sr^{2+} and 0.07 g of ^{15}N. The nutrient concentration in the 30 ml of solution injected was 0.16 m for Rb^+, Sr^{2+} and NO_3^--^{15}N, and 1.50 m for N. Finally, the holes were filled with the soil removed during drilling, respecting the original order of the soil horizons (da Silva et al., 2011).

2.4. Leaf sampling

Young fully expanded leaves were collected from the upper half of the tree crown of each pair of sampled trees (mean total height of 10.2 m for F+ and 7.2 m for F−). Rb^+ and Sr^{2+} concentrations and x (^{15}N) background values, as well as N, P, K and Ca concentrations were estimated for each pair of trees (48 trees in total) by sampling 10 leaves per tree before tracer application. Forty leaves (20 per labelled trees) were collected for each pair of trees, 70 days after tracer application (da Silva et al., 2011). The leaves were washed in deionized water, dried for 3 days at 65 °C and stored in sealed acrylic bottles until chemical and isotopic analysis; x(^{15}N) and N concentration were determined with a 20–20 Hydra mass spectrometer coupled to an automatic N analyser (ANCA-GSL, SERCON Co., Crewe, UK) from 10 mg of leaf powder dry matter. The concentrations of Rb^+ and Sr^{2+} were determined by inductively coupled plasma mass spectrometry (ICP-MS Agilent 7500ce; Agilent Technologies, Tokyo, Japan) after sample acid digestion using 0.5 g of leaf powder in a mixture of 5 mL of nitric acid (16 M) and 1 ml of perchloric acids (12 M). The limit of detection for Rb^+ and Sr^{2+} was 0.018 and 0.020 $mg\,kg^{-1}$ respectively. The relative standard deviation was less than 0.5%. The precision of isotope measurements was 0.001 x (^{15}N).

2.5. Root sampling and root traits measurements

Fine roots (diameter < 2 mm) were sampled on March 2016, at 22 months of age in each block, in F+ and F− treatments. Soil cores were collected at 4 positions randomly distributed around 4 trees of mean stand basal area (4 sampling positions per treatment and block) in the plots where tracers were injected (Fig. S1). At each sampling position, soil layers 0–20, 40–60, 130–170 cm were collected using a cylindrical auger with an inner diameter of 4.5 cm. In the same holes previously enlarged down to 170 cm depth using a tool designed to dig

deep holes (Germon et al., 2017), soil cores between 280 and 320 cm were collected using a cylindrical auger with an inner diameter of 6.0 cm, using the methodology described by Christina et al. (2011). Each soil sample was weighted in the laboratory and the soil water content was measured (drying 10 g of soil at 105 °C for 72 h). Each soil sample was homogenously mixed before being divided into two sub-samples representing approximatively 10% and 90% of the total soil mass. All the roots were recovered from both sub-samples of soil and carefully washed under tap water using sieves (with mesh sizes ranging from 1.19 to 0.50 mm) to be free of soil (Cornelissen et al., 2003). Living roots, identified from a white stele (central part of the root) and a good flexibility (ability to return to the original form when curved), were separated from dead roots that were discarded. The biomass of fine roots (with a length < 1 cm) was estimated in the subsample representing 10% of the soil. All the living fine roots (with a length > 1 cm in both soil samples) were scanned (800 dpi resolution) and root lengths, areas and diameter were estimated using the WinRHIZO Version Pro V.2009c software (Regent Instruments, QC, Canada). Fine roots were then dried at 65 °C for 72 h and weighed (± 0.1 mg). Dry masses, lengths and areas of fine roots were used to estimate the following root parameters: specific root length (SRL, ratio between length of scanned fine roots and their dry mass), specific root area (SRA, ratio between area of scanned fine roots and their dry mass) and fine root diameter (Maurice et al., 2010), for each soil layer, sampling position, treatment, and block (96 samples in total). Fine root mass density (RMD) was calculated by dividing root dry matter by dry weight of soil sample, and was then expressed in g of root per cm^3 of soil using soil bulk densities measured in each plot in a pit down to a depth of 3 m. Root length density (RLD) and root area density (RAD) were then calculated for each soil sample by multiplying RMD by SRL and SRA, respectively. The root tissue density was calculated as the ratio of mass per volume according to Wurzburger and Wright (2015).

2.6. RUP and SRUP calculations

Leaf concentrations of Rb^+ and Sr^{2+} and $x(^{15}N)$ were analysed for all samples of each treatment in the 3 blocks.

The RUP (Root Uptake Potential) was calculated according to da Silva et al. (2011):

$$RUP_{ip}\ (\%) = (T_{ip} - T_{i0})/[(T_{i10} - T_{i0}) + (T_{i50} - T_{i0}) + (T_{i150} - T_{i0}) + (T_{i300} - T_{i0})] \times 100 \quad (1)$$

where RUP_{ip} was the RUP of tracer i at depth p, T_{ip} was the foliar concentration (Rb and Sr) or $x(^{15}N)$ of tracer i at depth p, T_{i0} was the background value of foliar concentration (Rb and Sr) or $x(^{15}N)$, and T_{i10}, T_{i50}, T_{i150}, T_{i300} were the foliar concentrations or $x(^{15}N)$ of tracer i at the depths of 10, 50, 150 and 300 cm. For each tracer we divided the enrichment at a chosen depth by the enrichment at all depths in the same plot (for each treatment in each block).

The SRUP (Specific Root Uptake Potential) was calculated in each block for both treatments (da Silva et al., 2011):

$$SRUP_{ip}(\%\ cm^{-1}\ cm^3) = RUP_{ip}/RLD_p \quad (2)$$

where $SRUP_{ip}$ was the SRUP of tracer i at depth p and RLD_p was the fine root length density at depth p.

For each treatment, each depth, and each block, mean RLD values (n = 4) were calculated on the 10 cm above and below the depths of 10 cm and 50 cm, and on the 20 cm above and below the depths of 150 and 300 cm to account for the lower density of fine roots in deeper soil layers.

2.7. Statistical analysis

Generalized linear models (GLM) with binomial distribution and log-link function were used to (1) evaluate the effect of soil depth and fertilization treatment on fine root distributions, fine root traits, RUP and SRUP, and (2) compare the RUP and SRUP of the three tracers (NO_3^--^{15}N, Rb^+, Sr^{2+}) along the soil profiles. The significance of principal and interaction effects was conducted by nested model comparisons using likelihood ratio tests (D) (Zuur et al., 2009). For root distribution and root traits (n = 12), soil depth was introduced in the models as categorical variable, and post hoc Wald tests (W) were performed to assess differences between fertilization treatments or among tracers at the different depth levels. For RUP and SRUP (n = 3), depth was introduced in the models as continuous variable in order to increase model's degrees of freedom and statistical power, as recommended in small dataset analyses (Zuur et al., 2009). In such case, dependent variables were log-transformed when needed in order to linearize the relationships, and post hoc tests on the significance of depth effect were conducted using Student's t-tests (t). A statistical significance of the depth∗fertilization interaction on RUP or SRUP would indicate that their distributions along the soil profile differ between fertilization treatments. Homoscedasticity and normality of all the model's residuals were evaluated using Levene's tests and Kolmogorov–Smirnov tests, respectively. Analyses were performed using the R software version 3.2.2 (R development Core Team, 2013).

3. Results

3.1. Fine root distributions

Fine root mass density (RMD) was significantly influenced by depth (D = 321.8; P < 0.01) but not by fertilization. RMD decreased sharply from depths of 10 to 50 cm with mean values of 0.93×10^{-3} and 0.14×10^{-3} g cm^{-3}, respectively (Fig. 2a), and then slowly with mean values of 0.13×10^{-3} g cm^{-3} at 150 cm and 0.06×10^{-3} g cm^{-3} at 300 cm. We report a significant depth x fertilization interaction (D = 22.09; P < 0.01) that was ascribed to significantly higher RMDs at a depth of 150 cm for fertilized (F+) than non-fertilized (F−) trees (W = −3.19; P < 0.01), the opposite being found at a depth of 300 cm (W = 3.5; P < 0.001). Root length density (RLD) was significantly influenced by depth (D = 484.6; P < 0.01) and fertilization (D = 5; P < 0.05). Consistently to RMD, RLD decreased sharply from the depths of 10 to 50 cm and then gently down to the depth of 300 cm (Fig. 2b). The RLDs at the depths of 50, 150 and 300 cm accounted on average for 13%, 8% and 3% of the RLD at a depth of 10 cm. Mean RLDs across the sampled depths were 26% higher in F+ than in F−, with significant differences between treatments at 50 cm depth (W = −2.38; P < 0.02) and 150 cm depth (W = −2.13; P < 0.05). A significant depth x fertilization interaction (D = 7.58; P < 0.05) was the result of lower RLD for F+ than F− at 300 cm with mean values of 0.08 and 0.10 cm cm^{-3}, respectively.

Root area density (RAD) calculated for each sampled soil layer was significantly influenced by depth (D = 462.62; P < 0.01) but not by fertilization. RAD value at 10 cm was 10 times higher than the average of the three other depths (Fig. 2c). A significant depth x fertilization interaction (D = 22.09; P < 0.01) was the result of significant higher RAD at a depth of 150 cm for F+ than F− (W = −2.58; P < 0.02), the opposite being found at 300 cm depth (W = 2.8; P < 0.01).

3.2. Fine root traits

Specific root length (SRL) was significantly influenced by depth (D = 16.82; P < 0.01) and fertilization (D = 9.31; P < 0.01). SRLs values were on average 32 m g^{-1} at 10–50 cm and 23 m g^{-1} at 150–300 cm (Fig. 2d). SRL values were always higher in F+ than in F−, with a significant difference at a depth of 300 cm. We report a significant depth × fertilization interaction (D = 10.12; P < 0.02) that was ascribed to a higher variability in SRL with depth in F+ than in F−. Specific root area (SRA) was significantly influenced by fertilization (D = 4.84; P < 0.05) but not by depth. On average, SRAs were

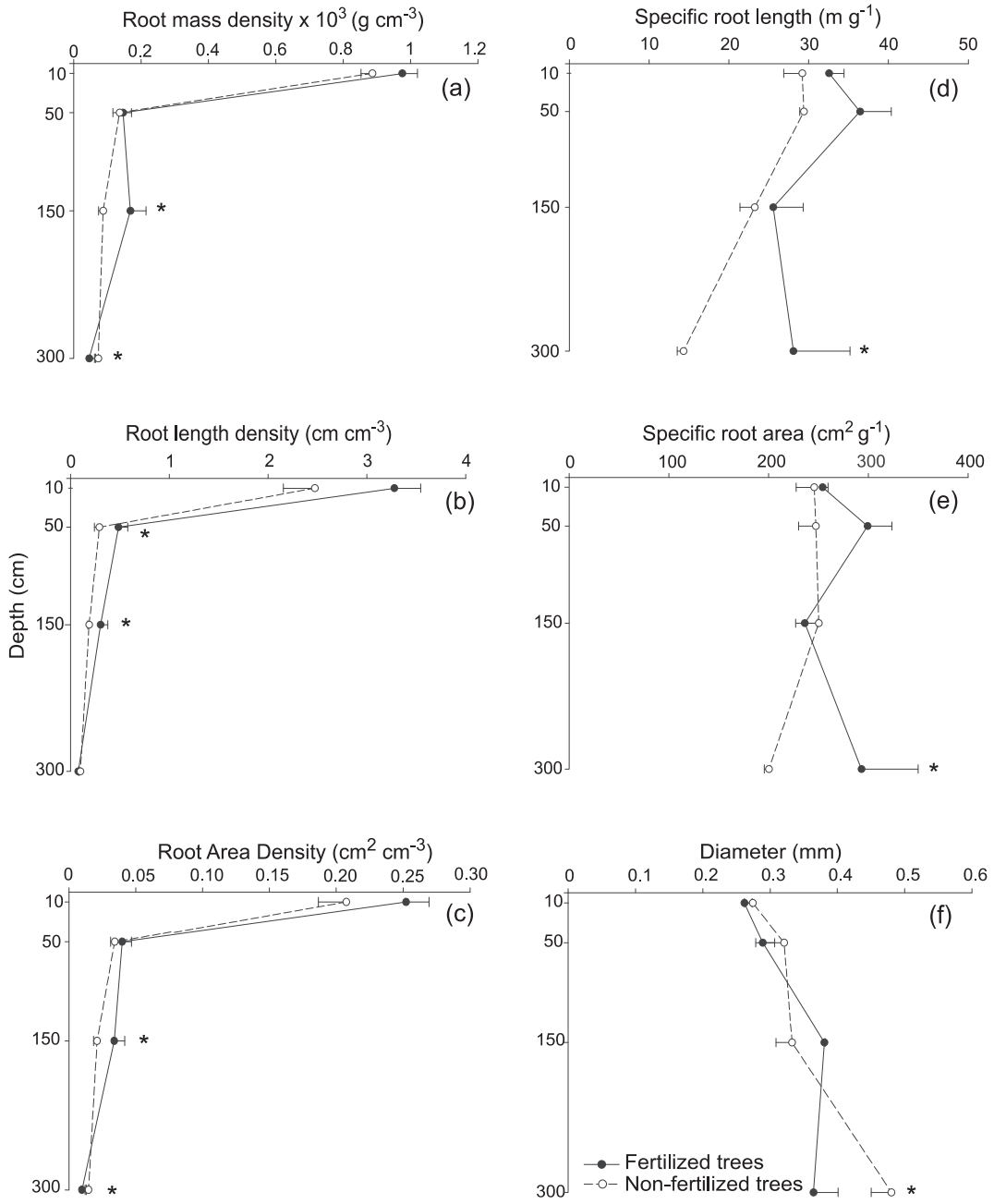

Fig. 2. Change with soil depth in root mass density (a), root length densities (b), root area density (c), specific root length (d), specific root area (e), and root diameter (f) for fertilized (F +) and non-fertilized (F −) trees. For each individual soil layer, * denotes a significant difference ($p < 0.05$) between F+ and F− at depths of 10, 50, 150 and 300 cm. Standard errors are indicated (n = 12).

270 $cm^2\ g^{-1}$ in F+ and 236 $cm^2\ g^{-1}$ in F− with a significantly higher value for F+ than for F− at a depth of 300 cm ($W = -3.45$; $P < 0.001$) (Fig. 2e). A significant depth × fertilization interaction ($D = 9.48$; $P < 0.05$) was the result of lower SRA for F+ than F− at a depth of 150 cm ($W = -3.45$; $P < 0.001$). Fine root diameter was significantly influenced by depth ($D = 44.65$; $P < 0.01$). A general trend of increase in root diameter with depth was observed for both treatments with average values of 0.29 mm at 10–50 cm and 0.39 mm at 150–300 cm (Fig. 2f). There was no significant effect of fertilization on fine root diameter. However, the diameters were, on average, 0.36 mm in F+ and 0.48 mm in F−, with significantly higher value for F− at a depth of 300 cm than F+ ($W = -4.24$; $P < 0.001$). A significant depth × fertilization interaction ($D = 11.63$; $P < 0.01$) was the result of lower diameter in F− at a depth of 150 cm ($W = 3.28$; $P < 0.001$). Fine root tissue density was significantly influenced by depth ($D = 26.294$; $P < 0.001$) with decreasing values down to a depth of 300 cm for both treatments (Fig. 5). There was no significant global effect of fertilization. However, significant lower value for F+ than F− at a depth of 300 cm was found. There was no significant interaction between depth and fertilization for fine root tissue density.

3.3. Relative uptake potential of tracers

The RUPs of NO_3^--^{15}N were significantly influenced by the depth of tracer injection for both treatments: fertilized trees ($t = -5.18$; $P < 0.001$) and non-fertilized trees ($t = -8.35$; $P < 0.001$). The RUPs of NO_3^--^{15}N was 16 times higher at a depth of 10 cm than 300 cm with mean values of 63% and 4%, respectively (Fig. 3a). The distribution of RUPs of NO_3^--^{15}N along the soil profile was not significantly influenced by fertilization. The significant interaction depth x

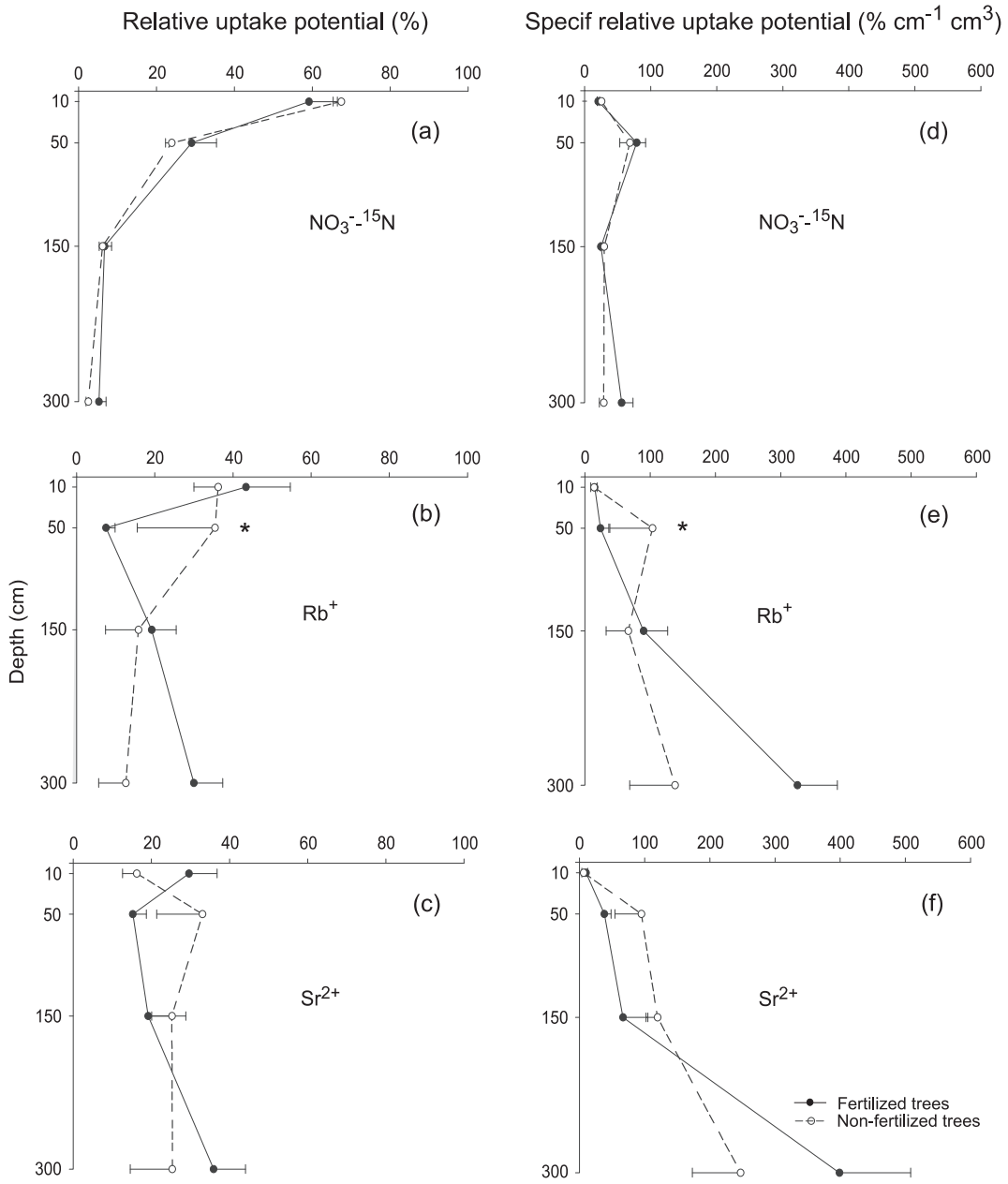

Fig. 3. Relative uptake potential (RUP) (a, b, c) and specific relative uptake potential (SRUP) (d, e, f) of NO_3^--^{15}N, Rb^+ and Sr^{2+} at depths of 10, 50, 150 and 300 cm for fertilized (F+) and non-fertilized (F−) trees. The RUPs and SRUPs were calculated 70 days after tracer injection. For each individual soil layer, * denotes a significant difference ($p < 0.05$) between F+ and F− at depths of 10, 50, 150 and 300 cm. The effect of depth is only significant for SRUP of Rb+ in F+. Standard errors are indicated (n = 3).

fertilization ($D = 5.28$; $P < 0.05$) was the result of lower RUP for F+ than F− at a depth of 10 cm with mean values of 59% and 67%, respectively, the opposite being found at a depth of 300 cm with 5% and 2%. The distributions of the RUPs of Rb^+ and Sr^{2+} along soil profile were uniforms (RUP values did not differ among soil layers) and not differ between fertilization treatments, with no significant interaction between the two factors. The RUP of Rb + was significantly higher at a depth of 50 cm for F+ than F− ($t = 2.35$; $P < 0.05$) (Fig. 3b). However, the RUPs of Rb^+ and Sr^{2+} were higher at a depth of 300 cm in F+ than in F− with mean values of 29% and 35% compared to 12 and 25%, respectively (Fig. 3b and 3c). The RUP was significantly higher for NO_3^--^{15}N than Sr^{2+} at a depth of 10 cm in F− ($W = 3.16$; $P < 0.01$), with mean values of 67 and 16%, respectively (Fig. 3a). The RUP of NO_3^--^{15}N was significantly higher than that of Rb^+ at a depth of 50 cm in F+ ($W = 3.41$; $P < 0.01$). The RUP was significantly lower in F+ and F− for NO_3^--^{15}N than Rb^+ at depths of 150 cm ($W = -2.67$; $P < 0.05$ and $W = -2.22$; $P < 0.01$, respectively) and 300 cm ($W = -4.42$; $P < 0.001$and $W = -3.78$; $P < 0.001$, respectively). The RUP was significantly lower in F+ and F− for NO_3^--^{15}N than Sr^{2+} at depths of 150 cm ($W = -2.8$; $P < 0.05$ and $W = -3.13$; $P < 0.01$, respectively) and 300 cm ($W = -4.87$; $P < 0.001$ and $W = -4.02$; $P < 0.001$, respectively). The RUPs of Rb^+ and Sr^{2+} were not significantly different, whatever the depth of tracer injection.

3.4. Specific relative uptake potential of tracers

The distribution of SRUPs for NO_3^--^{15}N along the soil profile was uniform (SRUP values did not differ among soil layers) and was not affected by fertilization. However, the mean values of the SRUPs of NO_3^--^{15}N were 37% higher at 10–50 cm (48% cm^{-1} cm^3) than at 150–300 cm (35% cm^{-1} cm^3) (Fig. 3d). The SRUPs of Rb^+ were significantly influenced by depths in F+ ($t = -4.29$; $P < 0.001$) with

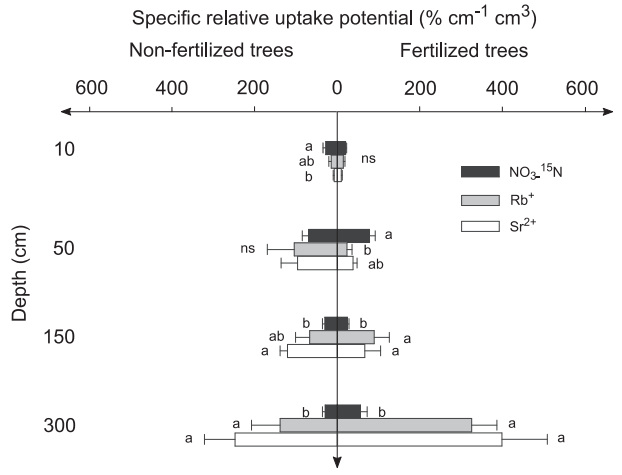

Fig. 4. Specific relative uptake potential (SRUP) of NO_3^--^{15}N, Rb^+ and Sr^{2+} at depths of 10, 50, 150 and 300 cm for fertilized (right) and non-fertilized trees (left). The SRUPs were calculated from determinations in leaves sampled 70 days after tracer injection. Standard errors are indicated (n = 3). Different letters indicate significant differences between tracers in each soil layer for each treatment ($p < 0.05$).

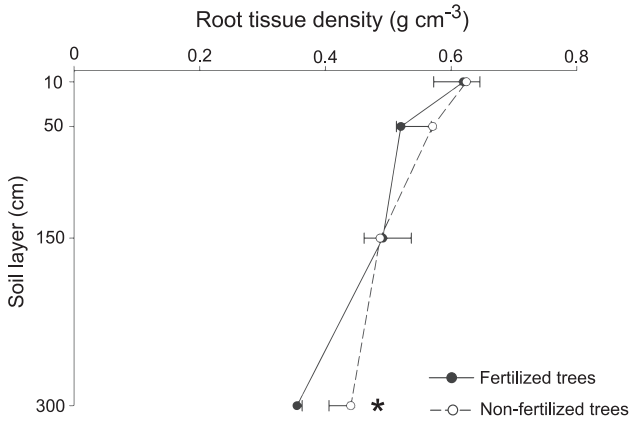

Fig. 5. Changes with depth in root tissue density for fertilized (F+) and non-fertilized (F−) trees. For each individual soil layer, * denotes a significant difference ($p < 0.05$) between F+ and F−. Standard errors are indicated (n = 3).

increasing values in line with the application depth, but not in F− (Fig. 3e). The mean values of the SRUPs of Rb^+ were 39% cm^{-1} cm^3 at 10–50 cm and 155% cm^{-1} cm^3 at 150–300 cm (Fig. 3e). The distribution of SRUPs along the soil profile for Rb^+ were marginally influenced by fertilization ($D = 3.18$; $P < 0.07$). The marginally significant depth x fertilization interaction for Rb^+ was mainly a result of a lower SRUP at a depth of 50 cm in F+ (24% $cm^{-1}cm^3$) than in F− (103% cm^{-1} cm^3), the opposite being found at a depth of 300 cm with mean values of 325 and 138% $cm^{-1}cm^3$, respectively. The SRUPs of Rb^+ was significantly higher at a depth of 50 cm for F+ than F− ($t = 3$; $P < 0.05$). The SRUPs of Sr^{2+} were significantly influenced by injection depth both for fertilized trees ($t = -5.32$; $P < 0.001$) and non-fertilized trees ($t = -3.46$; $P < 0.001$), but not by fertilization. The mean values of the SRUPs of Sr^{2+} values were 37% cm^{-1} cm^3 at 10–50 cm and 208% cm^{-1} cm^3 at 150–300 cm (Fig. 3f). There was no significant depth x fertilization interaction. However, the SRUPs values of Sr^{2+} at a depth of 50 cm was lower in F+ (38% cm^{-1} cm^3) than in F− (95% $cm^{-1}cm^3$), the opposite being found at 300 cm depth with mean values of 398 and 247% cm^{-1} cm^3, respectively. The SRUP of NO_3^--^{15}N was significantly higher than the SRUP of Sr^{2+} at a depth of 10 cm in F− ($W = 2.53$; $P < 0.05$) and higher than the SRUP of Rb^+ at a depth of 50 cm in F+ ($W = 2.69$; $P < 0.05$) (Fig. 4). The SRUP of Rb^+ was significantly higher than the SRUP of NO_3^--^{15}N at depths of 150 cm ($W = -2.93$; $P < 0.01$) and 300 cm ($W = -3.99$; $P < 0.001$) in F+ and of 300 cm in F− ($W = -2.94$; $P < 0.01$) (Fig. 4). The SRUP of Sr^{2+} was significantly higher than NO_3^--^{15}N in F+ and F− at depths of 150 cm ($W = -2.29$; $P < 0.05$ and $W = -2.61$; $P < 0.05$, respectively) and 300 cm ($W = -4.45$; $P < 0.001$ and $W = -4.02$; $P < 0.001$, respectively) (Fig. 4). The SRUPs of Rb^+ and Sr^{2+} were not significantly different, whatever the depth of tracer injection.

4. Discussion

4.1. Potential methodological limitations

Our assessment of the nutrient uptake capacity of tree roots in situ is associated with non-negligible methodological limitations, as stressed in previous studies (da Silva et al., 2011, 2008). Tracer availability for tree roots may have differed depending on depth and element properties. N microbial immobilization increases with soil organic resources (He et al., 2016) and NO_3^- may be preferentially bound in surface soils with higher organic matter content than in deep soil layer (Barrett and Burke, 2000). Moreover, N concentrations in soil solutions and turnover rates are likely to be higher in the topsoil than in deep soil layers (Voigtlaender et al., 2012). As a result, $N0_3^-$-^{15}N concentrations in soil solutions could have been lower in the topsoil than in deep soil layers. However, the dynamics of nutrient concentrations (in particular nitrate) monitored from planting to harvesting at different depths in *Eucalyptus* plantations at our study site showed that large amounts of nitrate in the topsoil move downwards in the soil profile (Maquère, 2008; Laclau et al., 2010). Large amounts of rainfall in this soil with a high sand content make unlikely a strong immobilization of ^{15}N in the top soil. In another study carried out in eucalypt plantations in Congo, Versini et al. (2014) showed that ^{15}N-labelled harvest residues led to a clear increase in ^{15}N in soil solutions sampled at the depths of 50 cm and 100 cm, which confirms the mobility of nitrate produced in the topsoil in these tropical plantations. Like $N0_3^-$-^{15}N, Rb^+ and Sr^{2+} uptake rates were not compensated for differences in their dilution in the soil or their sorption to soil particles depending on depth. Roots and mycorrhiza nutrient uptake zone may be overlapping more in the topsoil than in deep soil layers, which is another limitation of our approach that could contribute to increasing the SRUP of Rb^+ and Sr^{2+} with soil depth (Göransson et al., 2006, 2008). The SRUPs were significantly higher at a depth of 300 cm than at a depth of 10 cm for Rb^+ and Sr^{2+}, whereas the SRUP was not influenced by soil depth for the NO_3^--^{15}N tracer applied simultaneously at the same positions. This pattern for the mobile NO_3^- suggests that an overlap of the uptake zones around roots in the topsoil was unlikely to explain the large increase in the SRUP with soil depth observed for the analogues of K^+ and Ca^{2+}. Differences in the RLD between depths might have biased the estimation of the SRUP because tracers were added at a higher proportion for a given root length at depth than in the surface soil layers. Such bias should have been more pronounced for the highly mobile tracer NO_3^--^{15}N than for Rb^+ and Sr^{2+}. Moreover, SRUP was calculated as the ratio between RUP and fine root length per unit of soil volume with mean RLD estimated at each injection depth (in a soil layer of thickness 20 cm at injections depths 10 and 50 cm depth, and of thickness 40 cm at injection depths 150 and 300 cm). However, the actual volume of soil in which trees take up nutrients is not exactly the soil layer where RLDs were measured, which could slightly modify SRUPs values. A bioassay comparing *E. grandis* roots sampled at different depths would be useful in future studies to check the effect of depth on the element uptake capacity, as shown by Göransson et al. (2007) for excised roots of *Q. robur*.

A recent study found ectomycorrhizae associated with roots to 4 m depth in a nearby *E. grandis* stand (unpublished data) suggesting the need to gain insights into the role of mycorrhizal association in fine root functional specialization. Here, ectomycorrhizal root tips were also observed down to 3 m (data not shown). High ectomycorrhizal

colonization was found in both F+ and F− at 10 cm and 50 cm depth and could partly explain the comparable SRUP of NO_3^--^{15}N for fertilized and non-fertilized trees in the topsoil. A black morphotype (probably involving *Thelephora sp.* or *Tomentella* sp. fungi) was observed only in the upper layers while a yellow morphotype (probably involving *Pisolithus sp.* fungi) was found along the whole soil profile. The diversity of morphotypes between surface and deep soil layers might contribute to explaining the differences in functional fine root specialization. Despite these potential limitations, the marked differences in relative uptake rates between tracers for a given depth, between depths for a given tracer, and between treatments were unlikely to lead to erroneous conclusions on the fine root specialization of *E. grandis* at young stand stage depending on fertilization.

4.2. Fine roots functional specialization at young tree age

The results are in agreement with our first hypothesis of a functional specialization of fine roots for young *E. grandis* trees depending on soil depth, as previously observed for 6-year-old *E. grandis* trees in the same region (da Silva et al., 2011). The uptake of tracers by *E. grandis* trees down to 3 m deep at 20 months of age is consistent with the rapid soil exploration of fine roots that can reach a depth of 13 meters at 18 months of age in deep Ferralsols (Pinheiro et al., 2016). The sharp decrease in RMD and RLD with depth, as observed in other *Eucalyptus* plantations irrespective of trees age (Christina et al., 2011; Pinheiro et al., 2016), is consistent with the decrease with depth of the RUPs of NO_3^- shown for mature *E. grandis* trees (da Silva et al., 2011) and for other tropical tree species (Gathumbi et al., 2003; Soethe et al., 2006). Nitrate absorption was higher when RMD increased and this result would be consistent with regular nitrate absorption along the root as shown for *Pinus pinaster* (Plassard et al., 2002). However, the RUPs of Rb^+ and Sr^{2+} were not significantly affected by the injection depth, contrary to the observations of da Silva et al. (2011) at the harvesting age. This pattern suggests a marked specialization of deep *Eucalyptus* fine roots for uptake of the analogues of K^+ and Ca^{2+} at the beginning of stand rotation.

The SRUP distributions of Rb^+ and Sr^{2+} met the hypothesis of functional specialization of fine roots with significantly higher values at 300 cm depth than at 10 and 50 cm depths for both tracers. The higher SRUPs of Rb^+ and Sr^{2+} in deep soil layers than in the topsoil are consistent with higher fine root diameters, which might make it possible to increase the capacity of nutrient absorption through root cortex and associated mycorrhizas and transport capacity in root stele (Guo et al., 2008; Kong et al., 2017). Wang et al. (2015) showed that the diameter of first-order roots of 3 temperate hardwood species increased with depth and presented differences in xylem structure. Deep roots had thicker stele, wider maximum conduit and greater number of conduits per stele than shallow roots. These characteristics led to higher theoretical hydraulic conductivity of deep roots increasing their efficiency of water and nutrient transportation. Increase in fine root diameter with soil depth was also reported in other studies (e.g. Fort et al., 2017). The decrease in fine root tissue density (RTD) with soil depth might also lead to higher root specific hydraulic conductivity, as found for Australian tree and shrub species (Pate et al., 1995). Higher root specific hydraulic conductivities might increase the mass flow from the soil to the roots and then increase nutrient root uptake in deep soil layers relative to the topsoil (Costa et al., 2017). This pattern would be also consistent with larger diameters of xylem vessels in deep fine roots than in shallow fine roots observed in a nearby *E. grandis* stand (unpublished data), and in other studies (McElrone et al., 2004). The mean values of SRUP of Rb^+ and Sr^{2+} at 300 cm depth in the 20-month-old trees sampled here were 40% higher than in 63-month-old trees in the same soil type (sandy soil) with tracer injection at the same season (da Silva et al., 2011). This result suggests a greater specialization of deep fine roots to take up Rb^+ and Sr^{2+} over the early growth of eucalypt trees when nutrient requirements are higher than at the end of stand rotation (Laclau et al., 2010).

A specialization of fine roots in NO_3^--^{15}N uptake was also likely with higher SRUPs within the topsoil layers rather than at great depth. However, the mean values of SRUP for Rb^+ and Sr^{2+} at 300 cm depth (231 and 323% cm^{-1} cm^3, respectively) were 4.8 and 6.8 times higher than that of SRUP of NO_3^- at 10–50 cm (48% cm^{-1} cm^3), suggesting higher fine root specialization in the uptake of Rb^+ and Sr^{2+} in deep soil layers than of NO_3^--^{15}N in surface soil. This pattern could be also partly explained by the changes in fine root density distributions with tree age. RLDs were twice as high at 20 months of age in the surface layers as at 63 months (da Silva et al., 2011), whereas they were similar at 300 cm depth. The mean value of SRUP of NO_3^--^{15}N at 10–50 cm was 48% cm^{-1} cm^3 in our study and 69% cm^{-1} cm^3 at the end of stand rotation for the same season of tracer injection (da Silva et al., 2011). NO_3^- uptake probably does not change proportionally to RLD for high RLDs in the topsoil due to overlapping depletion zones (Andrews and Newman, 1970). The lower specialization of fine roots for NO_3^- uptake in the topsoil than in depth for Rb^+ and Sr^{2+} could be also related to pH variations along the soil profile. Garnett and Smethurst (1999) showed that low pH led to higher NH_4^+ uptake of *Eucalyptus nitens* Maiden fine roots (200% higher at pH = 4 than at pH = 6) but pH did not influence nitrate uptake. Here, pH values increased with depth (Table 1) as observed by Pradier et al. (2016) in a nearby eucalypt plantation. This higher potentiality of NH_4^+ uptake in the surface soil layers than in depth might then result in lowering NO_3^- uptake at 10–50 cm deep. The effect of depth on SRUP for a given tracer and the differences between tracers for a given depth in our study were marked and consistent for fertilized and non-fertilized trees. These results strongly support the hypothesis of functional specialization of fine roots for young eucalypt trees.

4.3. Fertilization enhances fine roots functional specialization

Fertilization at planting led to higher values of RMD, RLD and RAD along the soil profile for fertilized than for non-fertilized trees, except at a depth of 3 m. Applying fertilization at planting in *Eucalyptus* plantations established in sandy soil increases nutrient concentrations in soil solutions down to a depth of 3 m over the first 2 years after planting (Laclau et al., 2002, 2010; Silva et al., 2013). The changes in fine roots biomass with soil fertility are still controversial (Nadelhoffer, 2000). Most of studies reported a decrease in fine root biomass when soil fertility increases (Aber et al., 1985; Coomes and Grubb, 2000; Nadelhoffer, 2000; Vanninen and Makela, 1999; Yuan and Chen, 2010). However, no effect (Leuschner et al., 2004) or higher fine root biomass (George et al., 1997) were also observed when soil fertility increases, and there were greater mean RLD in high than low productive *E. grandis* stands close to the experimental area (Maurice et al., 2010). The differences between F+ and F− in RUPs of Rb^+ and Sr^{2+} did not mirror the changes in RMD, RLD and RAD in deep soil layers. At a depth of 300 cm, RMD and RAD values were lower for fertilized than for non-fertilized trees. However, at a depth of 300 cm RUPs of Rb^+ and Sr^{2+} were 140% and 40% higher for F+ than for F−, which suggests a higher ability of fine roots of fertilized trees to take up these two tracers. The higher RUPs in F+ than in F− could also be a result of higher mass flow to the roots due to higher transpiration of fertilized trees than non-fertilized trees (Hubbard et al., 2004; Battie-Laclau et al., 2016).

Consistent with our second hypothesis, the SRUPs of Rb^+ and Sr^{2+} are on average 136 and 61% higher in F+ than in F− at a depth of 300 cm, respectively, showing that fertilization likely increases the capacity of deep fine roots to take up K^+ and Ca^{2+} analogues. Significant lower RTD in F+ than F− in depth may lead to higher root specific hydraulic conductivity for fertilized trees and therefore to higher Rb^+ and Sr^{2+} uptake by unit of root length. This characteristic counterbalances the potential lower hydraulic conductivity resulting from lower root diameter at a depth of 3 m in F+ than in F−.

SRUP of Sr^{2+} at 3 m depth was also enhanced by NPK and

micronutrient addition, while calcium availability is not limiting the early growth of *Eucalyptus* trees in the study region, in contrast to N and K (Gonçalves et al., 2013). Therefore, improving the general nutrient status of *Eucalyptus* trees may affect several mechanisms of nutrient acquisition. Root specific hydraulic conductivity of *E. grandis* growing in pots was greater with high-localised availability of P than low P supply, leading to higher water and nutrients uptake (Costa et al., 2017). A study on the expression levels of nitrate and phosphate transporters genes in *A. thaliana* brought out evidences of a crosstalk between N and P transporters in regulating root nutrient uptake: e.g. AtPHT1;2, a major Pi transporter, was suppressed by nitrate starvation and induced by nitrate resupply (Bao et al., 2011). Rowe et al. (2008) showed that tissue P and K concentrations of *Calluna vulgaris* increased across a N deposition gradient, showing that acquisition of other plant nutrients was limited by N. The authors put forward the hypothesis that elevated P and K concentrations possibly resulted from improved acquisition due to additional root enzyme production or mycorrhizal activity (Rowe et al., 2008).

5. Conclusions and perspectives

Contrasting *E. grandis* potential uptake rates of NO_3^--^{15}N, Rb^+ and Sr^{2+} with depth at 2 years of age are consistent with previous results at harvesting age (da Silva et al., 2011). Functional root specialization increases with fertilization at planting time that likely improves potassium and calcium uptake in deep soil layers and contributes to very low amounts of cations lost by deep drainage in *Eucalyptus* plantations, even in highly fertilized stands (Laclau et al., 2010). Our findings reinforce the suggestion of these authors to apply fertilization once at planting for *Eucalyptus* stands established on deep soils with unhindered root development.

Further studies must be conducted to gain insights into the processes of fine root specialization. Mycorrhizal fungi that increase plant acquisition of N (Gobert and Plassard, 2008; Mayor et al., 2015), K and Ca (Garcia and Zimmermann, 2014; Jourand et al., 2014) could potentially impact the nutrient uptake of fine roots in depth. Fertilization could lead to change in microbial communities in depth, and then potentially to influence fine root specialization (Li et al., 2014; Gu et al., 2017). Variation of root hairs depending on depth and fertilization should also be an important process of functional fine root specialization of *Eucalyptus* trees, as root hair cells are highly selective for K (Maathuis and Sanders, 1996) and root hair elongation can increase with K concentrations within plants (Dolan, 2001). Getting insights into the potential uptake rate of P with soil depth would also be interesting to get a more global picture on fine roots specialization of *Eucalyptus* trees. Such studies could be conducted on *Eucalyptus* plants in controlled conditions using ^{32}P or ^{33}P tracers (Lehmann, 2003) or on excised roots (Göransson et al., 2007).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2018.03.018.

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