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Nitrogen Alters Initial Growth, Fine-Root Biomass and Soil Organic Matter Properties of a *Eucalyptus dunnii* Maiden Plantation in a Recently Afforested Grassland in Southern Brazil

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Abstract: Nitrogen (N) fertilization effects on *Eucalyptus* growth and soil carbon (C) stocks are still controversial. We set up an N fertilization experiment in southern Brazil to evaluate initial tree growth and changes in soil organic matter (SOM). Four N levels (24–Reference, 36, 48 and 108 kg ha^{-1} of N) were tested and tree growth was assessed during the first two years. Afterwards, representative trees were chosen to evaluate fine-root biomass (*FRB*) and its spatial distribution. Soil was sampled to a 40-cm depth and SOM was fractionated in Particulate (POM) and Mineral-Associated Organic Matter (MAOM) for C and N content, and $\delta^{13}C$ determination. Positive N effect on tree growth was seen only for tree height. N addition resulted in higher *FRB*. Changes in SOM were more expressive in top-soil layers. Overall, afforestation had positive effects on soil C. Increasing reference N dose resulted in higher C and N content in both SOM fractions. C and N dynamics were tightly correlated, especially in MAOM. Eucalypt-derived C was on average three-fold higher in POM. In summary, we showed that N fertilization may have positive but limited effects on tree growth, nevertheless it enhances fine-root biomass and C and N accumulation in SOM pools.

Keywords: land use change; nitrogen fertilization; *Eucalyptus* fine-root spatial distribution; SOM pools

1. Introduction

The growing demand for forest products over the last years and the great pressure to preserve native forests and mitigate increasing atmospheric CO_2 concentrations have increased the importance of planted forests. Planted forests cover more than 7.5 million hectares in Brazil and ca. 72% of this area is dominated by *Eucalyptus* spp., whose average productivities are the highest worldwide [1]. Southern areas in the country, particularly the Rio Grande do Sul State, are a new frontier of *Eucalyptus* expansion and the planted area in the State has increased 68% over the last 10 years [1,2]. It is a subtropical climate region and special attention should be given due to its particular characteristics [3,4].

Low temperatures and the risk of frost damage are primary concerns for eucalypt development in the Brazilian subtropical region. Therefore, tolerance to such conditions is essential for a successful planting, but tolerance usually happens at the expense of higher productivity. *E. dunnii* has shown good adaptability to southern Brazil climate conditions and is among the most planted species in the region [4–6]. However, there are few studies addressing this specie in the region or helping to identify suitable management practices to boost its productivity such as what has happened with other *Eucalyptus* spp. in other regions of the country [7–11].

The current high eucalypt productivity in Brazil was achieved mainly due to improvements in genetics and silvicultural practices. The relationship between productivity and fertilization is well known in agriculture and this is not different for forest plantations [10,12]. Forest growth is mainly limited by nitrogen (N) and phosphorus (P) in tropical regions [13]. However, the response of eucalypt plantations to N fertilization has been widely discussed and contradictory in the literature [11,14–21]. Some authors have shown positive responses to N fertilization [11,15,16], especially on initial growth phases [14], while others have shown no response to N supply, suggesting that the soil organic mineralizable N is the main source and sufficient to support tree demand [21–23]. Regarding below-ground growth, studies are far more scarce in associating N and root growth in a eucalypt ecosystem [24].

Nitrogen may also be important to sustain stable soil C formation, either by increasing biomass input and turnover, or by forming more complex compounds [25–27]. In Brazil, soil C stocks have shown different responses to afforestation [15,28–30] and might be dependent on former land use, edaphoclimatic conditions, tree species planted and tillage practices adopted [31]. Studies assessing the effect of afforestation on soil organic matter (SOM) properties in southern Brazil are scant [30,32,33] and are particularly important with afforestation expansion often taking place on a fragile ecosystem dominated by sandy-textured soils prone to wind and water erosion [34]. Sustainability of *Eucalyptus* forests is highly associated with SOM stocks, and thus understanding the consequences of eucalypt afforestation on SOM in these areas is crucial [13].

To investigate the effects of eucalypt afforestation and fertilization on initial growth and SOM properties, we set up an N fertilization experiment soon after the conversion of natural grasslands in the Pampa Biome in southern Brazil to *E. dunnii* Maiden plantations. We followed tree growth over the first two years of an expected 7-year rotation, evaluating above- and below-ground growth, and assessed early changes in SOM properties by fractionating in Particulate Organic Matter (POM) and Mineral Associated Organic Matter (MAOM)—two pools with different turnover times and sensitivities to land use change [35,36], and determining C and N content and the proportion of C derived from eucalypt (C_3)—using natural differences in ^{13}C isotope, associated with these SOM fractions.

2. Materials and Methods

2.1. Site Description and Experimental Design

The study site was located in Rio Grande do Sul State, southern Brazil, near the Brazil–Uruguay border (30°26′ S; 54°31′ W). The site is within the Pampa Biome, which is characterized by a grass domain with many herbs and shrub species co-occurring with the grass matrix. It is a new frontier of *Eucalyptus* plantation expansion in Brazil [1]. The site is located in a sub-tropical climate area (Cfa, Köppen classification), at ca. 150 m altitude and gentle slope, with mean annual temperature (MAT) of 18 °C and average annual rainfall of 1351 mm during the experiment years (Data from Brazilian National Institute of Spatial Research–INPE–S. Gabriel/RS station, located ~17 km away from experimental site at similar altitude).

Soils from the region are formed from sedimentary material mainly composed by arenite and siltite [34,37]. Soil was classified as Oxyaquic Hapludalf by the US Soil Taxonomy [38] or "Luvissolo Háplico Órtico típico" by the Brazilian Soil Classification System [39], with drainage constraints during wet season and occasional iron concretions (plinthite) occurrence in deeper layers (below 30 cm). It is

a relatively shallow soil, in which A and B horizons are limited to the uppermost ~55 cm of the soil profile. Soil chemical and physical characterization were performed in the whole treatment area, soon after eucalypt introduction, at 0–10, 10–20 and 20–40 cm depth. For each depth, intact soil core samples (one per plot, of a total 16 plots) were collected for physical characterization (Soil bulk density (ρ_b) and total porosity (TP)). For chemical and SOM characterization, five subsamples were collected per plot at each depth and mixed thoroughly to obtain one composite sample per plot at each layer. Average properties at the beginning of the experiment are summarized in Table 1.

Table 1. Average soil chemical, physical and organic properties at 0–10, 10–20 and 20–40 cm soil depth obtained at the beginning of the experiment in the whole treatment area (n = 16, for each depth), located in Rio Grande do Sul State, southern Brazil.

Depth	pH [(1)]	SB [(2)]	ρ_b [(3)]	TP [(4)]	Clay [(5)]	POM [(6)]			MAOM [(6)]			SOM [(7)]
						C	$\delta^{13}C$	N	C	$\delta^{13}C$	N	C:N
cm	-	$cmol_c\ dm^{-3}$	$g\ cm^{-3}$	$m^3\ m^{-3}$	$g\ kg^{-1}$	$g\ kg^{-1}$	‰	$g\ kg^{-1}$	$g\ kg^{-1}$	‰	$g\ kg^{-1}$	-
0–10	4.71	7.71	1.15	0.53	280	2.35	−14.75	0.14	19.22	−13.52	2.22	9.15
10–20	4.82	6.83	1.32	0.46	320	1.32	−15.74	0.07	14.37	−13.50	1.60	9.40
20–40	5.07	7.74	1.36	0.45	370	0.81	−16.11	0.03	10.79	−13.41	1.22	9.28

[(1)] pH determined in H_2O, 1:2.5 soil:water solution; [(2)] Sum of Bases, Ca^{2+} and Mg^{2+} determined in KCl 1 mol L^{-1}, K^+ and Na^+ determined with Mehlich-1 extractor; [(3)] Soil Bulk Density; [(4)] Total Porosity; [(5)] determined following Ruiz (2005) [40]; [(6)] Fractionation following Cambardella and Elliott (1992) protocol; C and N content and $\delta^{13}C$ determinations using an elemental isotope ratio mass spectrometer (EA-IRMS ANCA-GSL 20-20, Sercon, Crewe, UK); [(7)] Bulk SOM C:N ratio.

E. dunnii Maiden seedlings (100-days old) were planted in August 2012 using a 3.3 × 2.2 m spacing. Before planting, each planting row was subsoiled to a 40-cm depth and then prepared with zonal tillage, i.e., ridge tillage management was used on planting rows. Overall, planting row consisted of ~20 × 40 cm (height x width) ridges.

Amendment of soil for planting consisted of an initial 2 Mg ha^{-1} of lime broadcast in the whole area before soil preparation. Afterwards, 200 kg ha^{-1} of single superphosphate (18% of P_2O_5) were incorporated in the ridge area during tillage. At planting, 06:30:06 NPK fertilizer formulation (NPK + 0.6% B + 0.4% Zn) was applied on the planting row at 150 g $plant^{-1}$ rate. N fertilization at planting totaled 12 kg ha^{-1} of N. Using a completely randomized block design with four replications, the stand was divided in four treatments (totaling 16 plots) that consisted of four N rates in the form of Urea, applied as side-dress fertilization one year after planting: 12, 24, 36 and 96 kg ha^{-1} of N, totaling 24 (Reference dose, i.e., commercial application used by forest companies in the area), 36, 48 and 108 kg ha^{-1} of N applied. Fertilizer was manually applied as a solution well distributed in the crown area of each tree. P and K were also supplied as side-dressing fertilization, but in equal amounts for all treatments. Each plot contained 140 trees, being 20 trees in 7 different rows, occupying an area of ~1000 m^2. Understory vegetation development was prevented by periodic herbicide application.

2.2. Variables Assessed

2.2.1. Tree Growth

Tree height (H), diameter at breast height (DBH), dominant height (Hd) [41] and survival rate were measured at one year (1.08-year-old trees) after planting, i.e., right before the side-dressing fertilization, and at six months (1.54-year-old) and one year (2.33-year-old) afterwards. Basal area (G, $m^2\ ha^{-1}$) was calculated using DBH, tree mortality rate and plot area. All trees at each plot were measured and accounted to obtain the average tree growth per plot and treatment.

During the 1.54-year sampling, we also assessed leaf area index (LAI) and leaf N content. LAI was measured using a Li-COR LAI2000 plant canopy analyzer. For each plot, 30 LAI measurements were obtained, simultaneously with the above canopy light measurements that were done in an adjacent open field. Fully expanded leaves from average tree of each plot were collected for leaf N content. Leaves were collected in all positions (N, S, E, W) from the upper third of tree crowns and were mixed

to form one bulk sample for each treatment and replication. They were oven-dried at 60 °C and finely ground before sulfuric acid digestion for N determination by Kjeldahl method.

2.2.2. Fine-Root Biomass and Architecture

Fine-root (*Ø* < 2 mm) biomass (*FRB*) and its architecture were measured in July 2015 (2.95-year-old trees). We selected one representative tree per plot, i.e., each treatment and replication, for root sampling. Root sampling was carried out in approximately a quarter of the area (based on spacing) of each tree evaluated. For each tree, we sampled nine points in pre-defined locations (also based on spacing, see Figure 1) to a 40 cm depth, since we were studying a relatively shallow soil and almost 90% of fine-roots are expected to be concentrated in upper layers of the soil [42,43]. Points were collected in three different directions: in-row (*r*), inter-row (*i*) and a diagonal (*d*) at 45° between *r* and *i* axis (Figure 1). A 6-cm diameter auger was used for sampling.

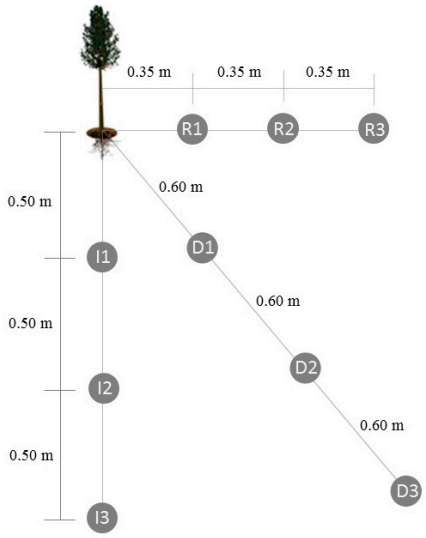

Figure 1. Representation of sampling design used for each representative tree. Area shown represents approximately a quarter of tree area (based on spacing). **R**: indicates row positions; **I**: indicates inter-row positions; and **D**: indicates diagonal positions at 45° between row and inter-row.

Samples were separated in 0–10, 10–20, 20–30 and 30–40 cm depth. For each point and depth, soil and roots were gently separated by hand. Roots were stored in plastic bags and taken to the laboratory for further cleaning, drying and weighing. Roots from one replication were stored in a 20% ethanol-water solution to keep fresh properties. In the laboratory, roots were thoroughly washed, and fine-roots were separated from the others with a sieve (2-mm mesh) and dried in a forced draft oven at 50 °C for 48–72 h to obtain total dry mass. Roots that were stored in the ethanol-water solution were washed and scanned for imagery at 300 dpi resolution before being oven dried. We processed root images with *Safira* software [44] to obtain average root length (*RL*), average root diameter, total root surface area and specific root area (*SRA*) (Supplementary material).

2.2.3. SOM Properties

Soil was collected in the same field campaign as root sampling, i.e., at 2.95-year stand age. Soil samples were randomly collected in five points (n = 5) per plot at 0–10, 10–20 and 20–40 cm depth. Samples were then grouped and mixed to build a composite sample of each soil layer per plot. Subsequently, samples were air-dried, crushed, 2-mm sieved and homogenized for SOM physical fractionation into Particulate Organic Matter (POM) and Mineral Associated Organic Matter (MAOM) [35]. Briefly, 5 g of soil were dispersed using 15 mL of a sodium hexametaphosphate (5 g L^{-1}) solution for 16 h at 200 rpm in a horizontal shaker. Afterwards, fractions were separated by a 53 µm

sieve by gently adding deionized water until the flush through the sieve was completely clear. All the material and water that flushed through the sieve (MAOM) was captured and dried in an oven for 6–8 days at 60 °C until completely dry. The material remaining on the sieve (POM) was removed by rinsing with deionized water again, recovered in a glass and dried in an oven for 4–5 days at 60 °C until dry.

After drying, POM and MAOM were then weighed and finely ground with a ball mill for C and N content and ^{13}C determination using an elemental isotope ratio mass spectrometer (EA-IRMS ANCA-GSL 20-20, Sercon, Crewe, UK). Reference gas was calibrated with Pee-Dee-Belemnite (PDB) certified standard for isotope signature calculations. The ^{13}C abundance in samples ($\delta^{13}C$) was calculated as follows:

$$\delta^{13}C(‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 100 \quad (1)$$

where $R = {}^{13}C/{}^{12}C$ ratio.

Based on $\delta^{13}C$, we could calculate, in each treatment, the proportion of C in each fraction that is derived from eucalypt plants (fC_{Euc}) as follows:

$$fC_{Euc} = \frac{\left(\delta^{13}C_f - \delta^{13}C_{Ref}\right)}{\left(\delta^{13}C_{Euc} - \delta^{13}C_{Ref}\right)} \quad (2)$$

where $\delta^{13}C_f$ is the $\delta^{13}C$ result obtained in SOM each fraction, $\delta^{13}C_{Ref}$ is the reference value of each fraction (initial value–Table 1), and $\delta^{13}C_{Euc}$ is the average $\delta^{13}C$ of eucalypt litter and roots sampled in the area.

Eucalypt litter was collected monthly using litterfall traps installed in the area. Each month, litterfall traps were cleared and the fallen litter was dried in an oven at 60 °C to obtain dry mass and litterfall rate. Each litterfall trap occupied an area of ~30 m^2 and accumulated litter from nine surrounding trees. The area under the traps was not used for soil or root sampling. The roots used for ^{13}C determinations were the same collected during root sampling in the representative trees. $\delta^{13}C_{Euc}$ was obtained by averaging roots and litter ^{13}C values based on total dry biomass of each component for each plot.

2.3. Data Analysis

Data were checked for normality and homoscedasticity for each variable and *ln*-transformed in necessary cases to meet normality assumptions. Split-plot two-way ANOVA and post-hoc Student LSD test, for tree variables (N and Time) and for SOM properties (N and Depth), were used to assess treatments effects. Regressions were fitted at each time in order to explain increasing N fertilization effects on tree growth (*DBH*, *H*) and fine-root biomass. Pearson correlation between above- and below-ground tree variables with SOM properties was also performed.

Spatial distribution of fine-roots across horizontal and vertical layers was represented using ordinary kriging of fine-root density (*FRD*). This method smoothes root distribution representation, by estimating *FRD* in areas where roots were not measured, based on the semi-variogram of variances of measured data, considering distance and direction. The semi-variogram model was adjusted according to the normality, stationarity, tendency and anisotropy of the samples, and its fitness was evaluated by a cross validation technique. All analyses were carried out in SISVAR® and R 3.03 software [45,46].

3. Results

3.1. Tree Growth

Trees presented similar growth pattern before side-dress fertilization (Figure 2). A change in the growth-response slope and a positive effect of N input on growth was seen 6 months and 1-year after

N fertilization (Figure 2). Nitrogen positively influenced tree *H* at 1.54 year (F = 10.639; p = 0.0026) and 2.33 year (F = 5.385; p = 0.0213). N influences on tree *DBH* were less expressive and non-significant in both times, although a nearly significant regression (p = 0.0822) could be used to explain *DBH* responses to increasing N at 1.54 year (Figure 2). Differences on basal area (*G*), tree survival, leaf area index (*LAI*) and leaf N content were all negligible and non-significant at all evaluated times (Table 2).

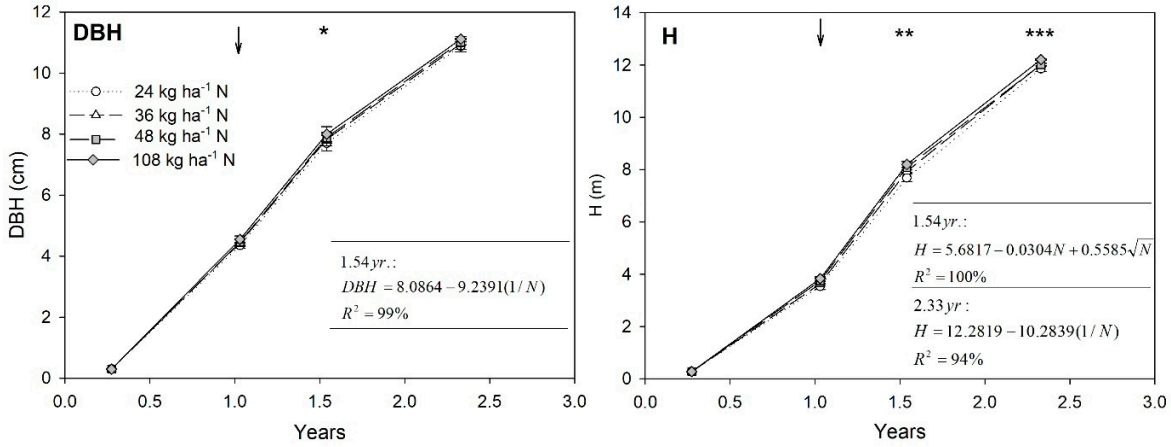

Figure 2. *E. dunnii* trees' average diameter at breast height (*DBH*, cm) and height (*H*, m) as a function of N level at each sampling age. Arrow indicates when treatments were applied. Vertical bars represent standard errors (n = 4). *, ** and *** indicate significance of regression (*Growth* × *N*) parameters at each age at 10, 5 and 1%, respectively.

Table 2. Effects of N dose on tree variables averaged for all trees at each treatment at 1.08, 1.54 and 2.33-year sampling.

Variables	-	Age (year)	Nitrogen Dose (kg ha^{-1})			
			24	36	48	108
DBH	cm	1.08	4.35 ^{a} (0.11)	4.44 ^{a} (0.12)	4.47 ^{a} (0.10)	4.55 ^{a} (0.11)
		1.54	7.70 ^{b} (0.25)	7.83 ^{a} (0.22)	7.89 ^{a} (0.18)	8.00 ^{a} (0.24)
		2.33	10.89 ^{a} (0.20)	10.97 ^{a} (0.18)	11.03 ^{a} (0.10)	11.11 ^{a} (0.10)
H	m	1.08	3.54 ^{a} (0.11)	3.64 ^{a} (0.09)	3.75 ^{a} (0.12)	3.83 ^{a} (0.08)
		1.54	7.69 ^{b} (0.15)	7.94 ^{a,b} (0.05)	8.09 ^{a} (0.10)	8.20 ^{a} (0.11)
		2.33	11.86 ^{b} (0.11)	12.02 ^{ab} (0.06)	12.02 ^{ab} (0.05)	12.21 ^{a} (0.03)
Hd	m	1.08	4.54 ^{a} (0.11)	4.71^{a} (0.08)	4.79 ^{a} (0.14)	4.79 ^{a} (0.18)
		1.54	8.83 ^{ab} (0.33)	8.51 ^{b} (0.26)	9.17 ^{a} (0.16)	9.15 ^{a} (0.27)
		2.33	12.64 ^{a} (0.23)	12.88 ^{a} (0.32)	12.94 ^{a} (0.16)	12.91 ^{a} (0.11)
Survival	%	1.08	94.82 ^{a} (1.71)	95.90 ^{a} (1.07)	95.71 ^{a} (1.34)	95.00 ^{a} (1.68)
		1.54	91.25 ^{a} (1.35)	92.68 ^{a} (1.03)	95.18 ^{a} (1.28)	92.14 ^{a} (1.62)
		2.33	92.14 ^{a} (0.23)	90.36 ^{a} (0.90)	91.96 ^{a} (1.63)	89.11 ^{a} (1.88)
G	m^2 ha^{-1}	1.08	1.55 ^{a} (0.13)	1.73 ^{a} (0.13)	1.82 ^{a} (0.18)	1.94 ^{a} (0.12)
		1.54	6.21 ^{a} (0.41)	6.29 ^{a} (0.32)	6.54 ^{a} (0.35)	6.61 ^{a} (0.47)
		2.33	12.49 ^{a} (0.41)	12.65 ^{a} (0.23)	13.17 ^{a} (0.30)	13.14 ^{a} (0.31)
LAI	m^2 m^{-2}	1.54	2.50 ^{a} (0.31)	2.72 ^{a} (0.13)	2.73 ^{a} (0.23)	2.78 ^{a} (0.30)
Leaf N	g kg^{-1}	1.54	21.68 ^{a} (1.32)	22.08 ^{a} (1.52)	22.33 ^{a} (0.99)	23.07 ^{a} (1.31)

DBH: Diameter at breast height; *H*: Height; *Hd*: Dominant height; *G*: Basal area; *LAI*: Leaf area index; *Leaf N*: Foliar N content. Standard errors (n = 4) are presented inside parentheses. Averages within each sampling period (i.e., each row) followed by same lower-case letter do not differ at 5% by LSD test.

3.2. Fine-Root Biomass (FRB) and Spatial Distribution

Fine-root biomass differed among N doses (F = 10.002; p = 0.0032). *FRB* responded positively until ~60 kg ha^{-1} of N, whereas the addition of 108 kg ha^{-1} of N resulted in the lowest *FRB* observed (Figure 3).

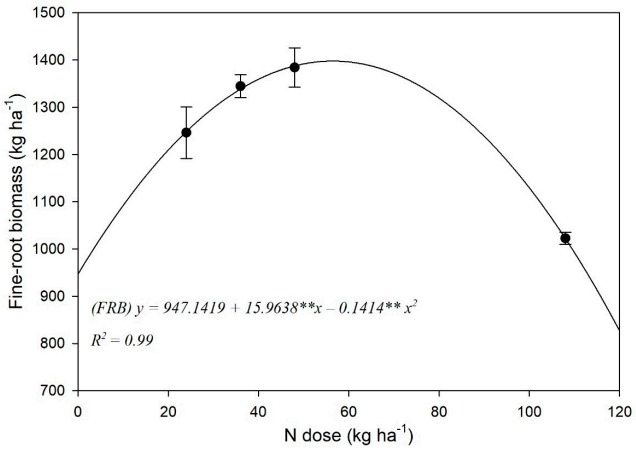

Figure 3. Fine-root biomass (*FRB*) until 40 cm depth at 2.95-year-old *E. dunnii* Maiden plantation as affected by N fertilization. Vertical bars represent standard errors (n = 4). ** indicates parameter is significant at 5%.

Fine-root density (*FRD*) showed a high heterogeneity along soil profile, and there was both horizontal and vertical anisotropy in root distribution. Root spatial distribution varied among the different N doses tested (Figure 4). The 0–10 cm layer had more homogeneous fine-root spatial distribution (Figure S1). The 108 kg ha^{-1} of N dose resulted in lower *FRD* and lower heterogeneity in fine root spatial distribution. Tillage seemed to change root distribution pattern along soil surface and profile (Figure 4). Row region tended to present higher *FRD* and *FRD* seems to increase along soil profile at planting row. Inter-row and diagonal positions presented higher *FRD* in soil top layers, and the highest *FRD* was observed at 10–20 cm layer.

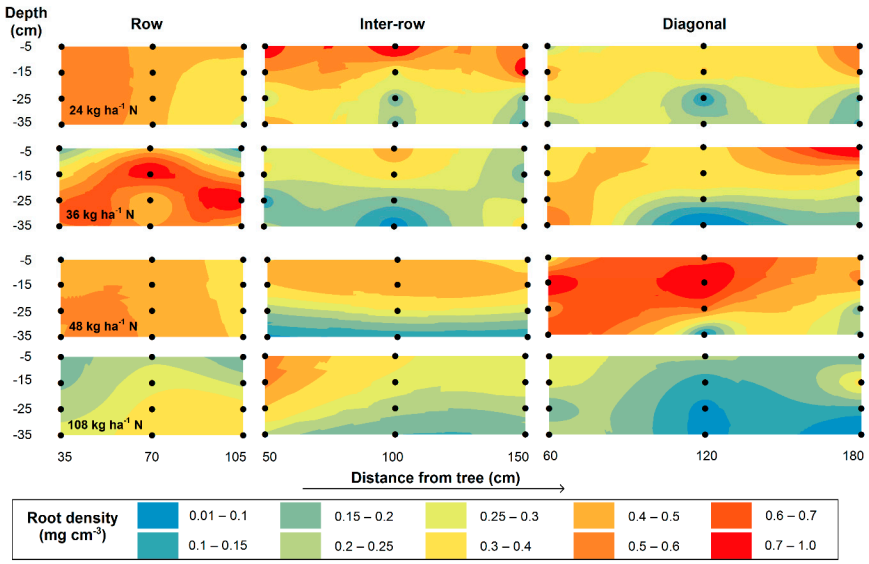

Figure 4. Ordinary kriging of average fine-root density (*FRD*) distribution of 2.95-year-old *E. dunnii* Maiden along soil profile in the three positions evaluated. Trees are placed in the top left corner of each figure. x axis represents increasing distance from tree in each position, and y axis represents depth.

3.3. C and N Associated with SOM Fractions as Affected by N Level

Nitrogen fertilization altered C and N associated with POM and MAOM. N effects were limited to 0–10 and 10–20 cm soil layers and different between fractions (Figure 5). Reference N level (24 kg ha^{-1}) resulted in the lowest C-POM and N-POM at 0–10 cm layer. Overall, 48 kg ha^{-1} of N was the dose that resulted in the higher C and N contents: the highest C-POM at 10–20 cm layer and the highest C-MAOM and N-MAOM at 0–10 cm layer. Contribution of eucalypt-derived C varied between SOM fractions, but in each fraction similar contributions were observed across the soil layers evaluated. C_3-derived C were on average two or three-fold higher in POM, ranging from 9 to 19% in C-POM, and from 0 to 7% in MAOM fraction. Reference N dose resulted in the lowest eucalypt-C proportion in both SOM fractions at 0–10 cm layer. Also, 108 kg ha^{-1} of N induced the lowest eucalypt-derived C in MAOM at 10–20 cm layer. $\delta^{13}C$-POM became more negative downward soil profile, whilst $\delta^{13}C$-MAOM presented an overall opposite behavior. Differences in $\delta^{13}C$-POM and $\delta^{13}C$-MAOM in deeper layers were small and often negligible.

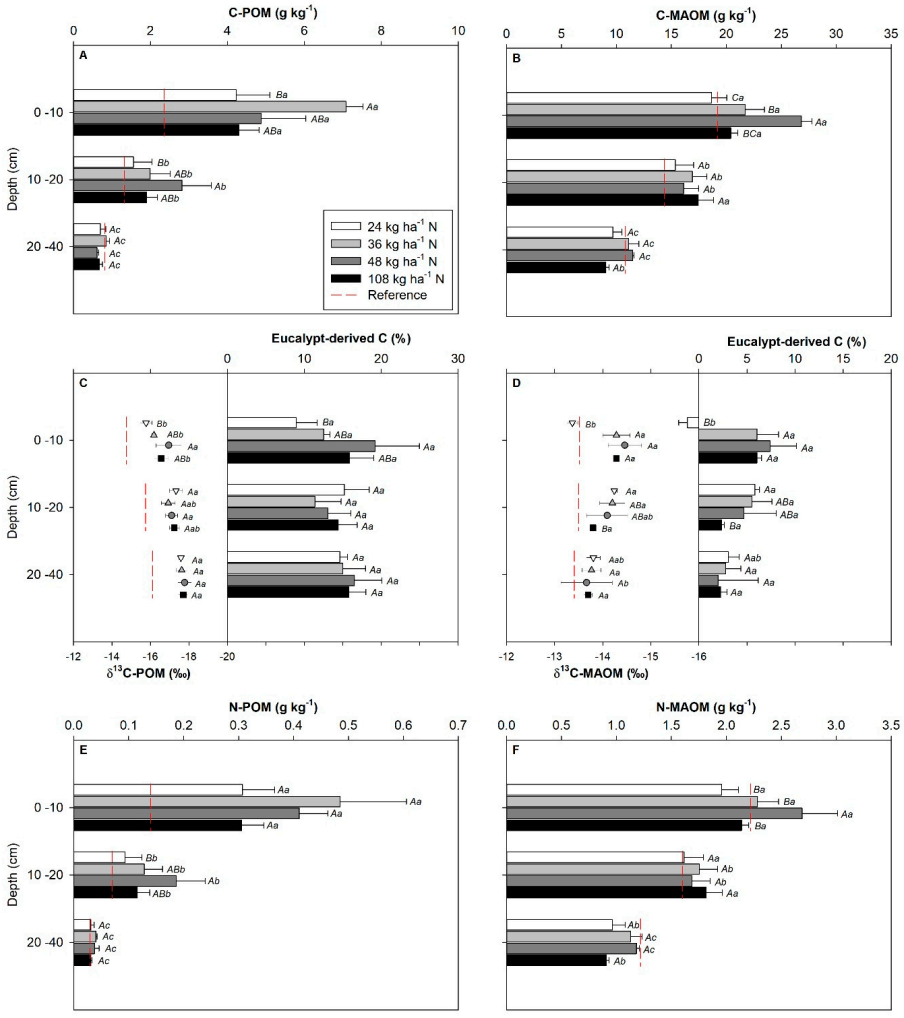

Figure 5. N fertilization effects on *C* (g kg^{-1}, (**A**,**B**)), $\delta^{13}C$ (‰, bottom *x* axis) and eucalypt-derived C (%, top *x* axis) (**C**,**D**), and N (g kg^{-1}, (**E**,**F**)) content of SOM fractions (POM—(**A**,**C**,**E**); and MAOM—(**B**,**D**,**F**)) at 0–10, 10–20 and 20–40 cm soil depth at 2.95-year-old eucalypt plantation. Reference lines (in red) indicate site properties at experiment beginning (same as found in Table 1). Error bars represent standard error (n = 4). Bars followed by same upper-case letter inside each depth and same lower-case letter inside each N level do not differ at 5% by LSD test.

4. Discussion

4.1. Tree Growth

Eucalypt growth response to N has been widely debated. Mineralizable N and the efficient internal N cycling are considered to suffice their fast growth [47,48]. Seldom responses of eucalypt growth to N fertilization are found in literature and they are usually limited to the first two years, while canopy is still in formation or under N limiting situations [11,14–20]. Here, tree height responded positively to N fertilization during the two-year evaluation, even with relatively high initial soil N content and low soil C:N ratio (Table 1). N effects on growth were greater at 1.54 year age, i.e., six months after treatment application, and a change in the slope of growth curves could be seen (Figure 2). Although this slope change could be attributed to seasonality (beginning of spring), the significance of fitted Growth × N regressions for *DBH* and *H* ($p = 0.0822$ and $p = 0.013$, respectively), and ANOVA significance ($p = 0.0026$) for *H*, indicate these variables were positively influenced by N, and the fitted regressions explain how they responded to N fertilization. At 2.33 year, only *H* was positively influenced ($p = 0.0213$). This faster initial growth obtained with increases in N relative to reference dose is important as it may result in further earlier canopy closure, lower weed competition and higher stand uniformity by overcoming any possible spatial variation of soil constraints [10,24].

Differences in eucalypt *LAI* and leaf nutrient content due to fertilization may respond to a lower extent than growth [17]. Indeed, here, the highest N dose resulted in the highest measured *LAI* and leaf N content (Table 2), although differences were negligible and non-significant among treatments. *LAIs* smaller than 3 m^2 m^{-2} as found here have already been reported for eucalypt tropical plantations [20,49]. It is still considered low, but it is expected to increase as *LAI* tends to peak when eucalypt forest is around 4-year-old [50].

4.2. Fine-Root Biomass and Spatial Distribution

Total amounts of fine-root biomass found here are in agreement to other studies that have addressed eucalypt roots of similar age plantations [24,51,52] or older [53,54]. In the present study, the highest N dose resulted in the lowest *FRB* observed (Figure 3). The higher N availability may have resulted in needless root expansion in the highest dose, causing a likely downward trend in the proportions of carbohydrates allocated to belowground biomass [24,51,55].

In a study with *Eucalyptus grandis* plantations in Brazil, N fertilization (120 kg ha^{-1} N) had no effect on fine root-growth within the upper 30 cm soil depth [24]. The authors argued this result was consistent with the observed limited effect of N on aboveground tree growth, in contrast to what we found here. In another study, in a Spodosol located in a Mediterranean type climate area, it was also found that there were no differences in eucalypt fine-root production due to fertilization, but an increase in *FRB* was observed when fertilization was combined with water supply [51]. These results indicate the responses of fine-root biomass to N fertilization are varied and dependent on tree species and environmental and edaphic properties.

Anisotropy in eucalypt fine-root distribution is commonly observed in the literature [42,56,57]. Both horizontal and vertical anisotropy in fine-root growth were noticed in our study and might have resulted in some of the fine-root clustering observed (Figure 4). Fine-root spatial distribution presented a strong behavior likely being driven by ridge tillage, i.e., concentrating in the row area. Overall, eucalypt fine-roots tend to concentrate in upper soil layers, and decrease sharply with depth [42,43,53,54,56,58]. Here, roots growing on the ridge increased with depth. At the planting row position, soil was subsoiled and a ridge of ~20 cm height was created that might have influenced the pattern of fine-root distribution. Soils tend to be more susceptible to temperature and moisture variation in shallower layers in a row area due to aggregate breakdown with pre-planting operations, driving roots downward. Furthermore, trees probably invested in deeper roots in the ridge (row) area due to the elevated density in surrounding areas and these are possibly the reasons why more roots could be found in the planted row below 20 cm depth (Figure 4). In the other positions, *FRD* peaked in

10–20 cm layer and then decreased. This is particularly interesting due to the higher soil density of this layer in comparison to the uppermost soil layer (shown in Table 1) and is probably a consequence of physical obstructions, e.g., plinthite, found in deeper layers, resulting in the accumulation of roots in 10–20 cm layer at inter-row and diagonal areas.

Some authors argued that fertilization or irrigation may alter eucalypt fine-root distribution, causing a higher concentration of fine-roots in planting row areas near the stump [24,42,59,60]. This could be a reasonable explanation for the higher *FRD* in planting row found in our study, however we could not see a decreasing gradient in *FRD* from planting row to inter row or diagonal positions (Figure 4). Thus, although observing differences in *FRB* and fine-root spatial distribution among N-levels, we believe N fertilization had little or no effect on fine-root spatial distribution, and suggest that tillage and soil properties might be the most responsible factors for the variations observed in fine-root spatial distribution along soil surface and profile.

4.3. SOM Properties as Affected by N Levels

C and N associated with SOM fractions presented similar responses to N fertilization rates (Figure 5). C and N dynamics were tightly correlated in both POM ($r = 0.84$, $p = 0.0001$) and MAOM fraction ($r = 0.92$, $p < 0.0001$).

Overall, C and N accrual were observed 2.95 year after eucalypt afforestation in comparison to the beginning of the experiment (Figure 5). Accrual occurred mainly in the top soil layer, especially in POM fractions, whereas deeper layer (20–40 cm) remained unchanged or presented lower C and N content. Here, soil C accrual, particularly in POM fraction, seemed rather fast and it is hypothesized that it might be a consequence of the low soil C status of the natural grassland before afforestation [29,33,61], the greater grassland-litter input due to vegetation substitution and a likely imbalance on decomposers caused by changes in litter type input [62,63]. Eucalypt afforestation is likely to increase the proportion of lignin-derived [30] and nonpolar alkyl C compounds [32] in the soil. In early stages of afforestation, the increase in the proportions of these compounds might lead to their accumulation in non-stabilized SOM fractions, since less adapted soil microorganisms to this new fresh material are expected to be found. We believe three hypotheses may be considered to explain this soil C accrual and the differences observed among N levels: (a) the differences in *FRB* production and turnover, since *FRB* correlated positively with C-POM ($r = 0.58$, $p = 0.0186$); (b) N addition may have inhibited lignin-degrading enzyme activity [64,65]; and/or (c) addition of N might have helped to overcome any eucalypt litter N limitation and resulted in earlier establishment of microbial community structure [62].

Both $\delta^{13}C$-POM and $\delta^{13}C$-MAOM became more negative, with $\delta^{13}C$-POM changing faster. Eucalypt-derived C ranged from 9 to 19% in C-POM, and from 0 to 7% in MAOM. Increases in the reference N dose resulted in more eucalypt-derived C in both fractions, particularly in the top-soil layer. Interestingly, while top soil layer presented higher C-POM content with eucalypt afforestation, it also presented a less negative $\delta^{13}C$-POM (Figure 5). This could be attributed to either the initial lower (more negative) $\delta^{13}C$-POM in deeper layers, or to the higher C content and greater litter-C from the former vegetation in upper layers, creating a dilution effect. The slight decrease of C-POM in the 20–40 cm layer observed, despite similar eucalypt contribution in comparison to upper layers, is intriguing. The overall lower root growth and aboveground litter contribution, combined with the probable slower turnover in deeper layer would be the first option to explain this decrease in C-POM content. But we speculate, since similar eucalypt-derived C is observed, if this response might not be evidence that fresh root input may destabilize old deep soil C, balancing C inputs and losses, and preventing net gains in soil C content [66].

Afforestation effects on soil C are largely debated in the literature [29–31,67,68]. The positive effects of N on soil C pools shown here are in disagreement with other authors who found no effect of N fertilization on soil C pools under eucalypt plantations [15,68]. Much higher N doses (300–1600 kg ha^{-1} of N) were tested on the development of first-rotation eucalypt forest in Hawaii [68], but no evidence of N altering soil C accrual was found because losses of old C_4-derived C offset C_3-derived inputs

throughout an 8-year rotation. Here, after 2.95 year, we observed a positive effect on soil C and N and a depletion in $\delta^{13}C$ particularly when the reference N dose was increased (Figure 5). The effects of eucalypt afforestation and N input on SOM dynamics observed in the early stages of the rotation may persist longer and be similar to those observed at the end of a rotation [67,68]. Hence, this early assessment could be valuable information to define proper stand management practices. Although effects of N addition observed here can change through the end of rotation, we hypothesize that effects might be even enhanced as a result of combined mineral and organic N effects on soil C pools [26]. Thereby, we argue that despite little or infrequent responses of eucalypt growth to N fertilization found here or elsewhere [14,47], the proper management of N fertilization in the evaluated areas could increase soil C and N stocks, and may help avoid excessive soil N mining in successive rotations.

5. Conclusions

Our results show that N fertilization alters tree growth, fine-root biomass and SOM fractions in early stages of eucalypt plantations in southern Brazil. A positive change in the slope of growth curve with N addition was observed, but differences were only significant for tree height. Fine-root biomass and SOM fractions were also positively influenced by increasing N supply. The 48 kg ha^{-1} of N dose resulted in the highest fine-root biomass and stable soil C and N content. Our results indicate that small adjustments in the current N fertilization regime adopted in the region may offer higher productivity and sustainability in early stages of *E. dunnii* plantations in southern Brazil.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/9/2/62/s1, Table S1: Root variables obtained from root imaging and Safira® software processing; Figure S1: Ordinary kriging of average fine-root density (*FRD*) distribution of 2.95-year-old *E. dunnii* plantations, in the four N rates tested, across the four depths evaluated (0–10: −5 cm; 10–20: −15 cm; 20–30: −25 cm; 30–40: −35 cm).

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