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Species richness, forest age, and litter affect SOC stock

DRIVERS OF SOC STOCK IN NATURALLY RESTORED SUBTROPICAL FORESTS AND MONOCULTURES OF NON-NATIVE TREES

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Abstract: In subtropical landscapes, forested areas previously occupied by agriculture have been naturally restored or planted with non-native species monoculture. Those two strategies are important for sequestering carbon in biomass and soil. However, the effects of plant diversity on soil carbon stock are still a matter of debate. The balance between carbon input and output processes can determine the potential for soil carbon storage. Here, we investigated if naturally restored subtropical forests differ from planted tree monocultures (*Eucalyptus grandis* W. Hill ex Maiden and *Pinus elliottii* Engelm) in terms of soil carbon stock, and whether factors such as richness, forest age, litter production, and quality (C:N ratio and lignin content) can drive soil organic carbon (SOC) stocks in these forests. The most richness and the old natural fragment had higher SOC stock, and natural fragments had a higher litter production but a lower litter C:N ratio than monocultures. Monocultures did not differ in richness, SOC, litter production, and C:N ratio.

Forest age and richness has a positive effect on the SOC stock, while the litter C:N ratio had a negative effect. Our findings indicate that, even in small forest fragments, the higher richness associated with the longer time of natural regeneration drives soil C storage. So, our results highlight the lower potential of forest plantations in restoration actions aimed at SOC stock recovery, reinforcing policies that encourage natural forest regeneration.

Keywords: C:N ratio, conservation strategies, lignin, natural regeneration, species richness

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INTRODUCTION

The conversion of forests into other land uses directly impacts diversity (Turner 1996, Newbold *et al.* 2015), and deforestation and fragmentation substantially increases carbon emissions in tropical regions (Brinck *et al*. 2017, Maxwell *et al*. 2019). Reducing the emission of greenhouse gases and promoting carbon sequestration are goals of several international agreements (e.g., Aichi Targets, REDD +, and the Paris Agreement) among nations to combat climate change. Forest restoration has become an essential part of environmental policies, as it contributes conserving diversity and maintaining ecosystem services (Overbeck *et al*. 2013). Currently, one of the most discussed alternatives for carbon recovery and diversity is via forest restoration, especially through natural regeneration (Capellesso *et al*. 2021, Jakovac *et al*. 2024).

Brazilian Atlantic Forest is one of the most threatened biomes in the world and a biodiversity hotspot (Myers *et al*. 2000), with a remaining coverage of only 28% of its original distribution (Rezende *et al*. 2018). The restoration strategies applied to the Atlantic Forest, especially in traditional areas marked for agricultural exploration, comprise mainly natural regeneration but also tree plantation, both in small forest fragments (Ribeiro *et al*. 2009, Mariano & Christianini 2016). Forests in the natural regeneration process have increased considerably in subtropical landscapes since 1990 due to the abandonment of areas used for agriculture (Rovani *et al*. 2019). Also, many unproductive areas have been planted with non-native tree monocultures. In Brazil, tree plantations encompass over 3 million hectares (PEVS 2020). As of 2019, Brazil

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had approximately 10 million hectares dedicated to monocultures, with eucalyptus covering 7.6 million hectares and pinus covering 2 million hectares (PEVS 2020).

The monocultures negatively affect SOC stocks compared to native forests and/or multispecies plantations, mainly due to higher litter production and management practices (Gatti *et al*. 2019). SOC also decreased in *Pinus elliottii* Engelm., and *Eucalyptus* spp. plantations due to the low diversity, which results in lower litter quality with a higher carbon-nitrogen (C:N) ratio (Kaschuk et al. 2011, Gatti *et al.* 2019, Teixeira *et al*. 2023). This influences leaf decomposition (Parton *et al*. 2007, Cuchietti *et al*. 2014) and the efficiency of microorganisms in metabolizing and stabilizing SOC (Cotrufo *et al*. 2013, Cotrufo *et al*. 2015, Li *et al*. 2020).

The subtropical forest is highly diverse (Bordin *et al*. 2021, Capellesso *et al*. 2021). It shows great potential for carbon sink in different succession stages (Bordin *et al*. 2023), which is attractive in terms of environmental policies for forest restoration (Vibrans *et al*. 2022). Moreover, in subtropical forest fragments, SOC values of approximately 84 Mg C ha^{-1} have been observed, which is 140% higher than the global median observed, expressing the high potential for soil carbon storage in these areas (Teixeira *et al*. 2023).

Despite the important role of subtropical forests in the carbon cycle (Pan *et al*. 2011), few studies have investigated whether tree diversity is correlated with soil carbon stock, especially in Brazilian subtropical forest fragments undergoing natural regeneration. Most studies examining the relationship between diversity and soil carbon stock in subtropical forests evaluated areas with small differences in species numbers or planted mixed tree species (Gamfeldt *et al*. 2013, Liu *et al*. 2018, Li *et al*. 2020).

Here, we aimed to compare the SOC stock in naturally restored forests and non-native tree monocultures (*Pinus* and *Eucalyptus*) and explore if the forest age (time of natural regeneration or planting), richness, and litter can drive the SOC stock in these small subtropical forest fragments.

MATERIALS AND METHODS

Study areas

This study was performed in Southern Brazil, in a highly fragmented area belonging to the southern portion of the Atlantic Forest biome in the Atlantic Araucaria Forest domain (Oliveira-Filho *et al*. 2015). Approximately 85% of this area is an agricultural matrix (Rovani *et al*. 2019). The climate is subtropical, with a mean annual precipitation of 1,800 mm and a mean annual air temperature of 18 ºC (Alvares *et al*. 2013), with monthly variations from 13 to 22 ºC. The soil in the studied fragments is a Rhodic Ferralsol by FAO/Unesco or Rodhic Hapludox by US Soil Taxonomy.

Four fragments were selected, two natural fragments with secondary succession (NF1, NF2, natural regeneration) and two non-native forests with monocultures (EM, PM). All evaluated areas are linked to a company's off-gassing compensation project and were previously used as agricultural land. NF1 and NF2 are 34 km apart. EM and PM are connected (27° 37' 42.28" S and 52° 21' 30.70" O) and located 36 km apart from NF1 (27º 28' 39" S and 52º 31' 45" W) and 10 km from NF2 (27° 33' 12.62" S and 52° 7' 28.85" W). The land use change was due to abandonment followed by tree plantation or successional trajectories. The age of the selected areas was assessed by analyzing images from Landsat TM5 and Landsat Oli8 satellites.

The first native forest fragment (NF1) has 19.4 ha with a density of 3,530 ind ha^{-1}, and the natural regeneration occurred between 1986 and 2013 (approximately 27 years of restoration). The second native forest fragment (NF2) has 52.3 ha with a density of 2,190 ind ha^{-1}, and the natural regeneration occurred between 2001 and 2013 (approximately 12 years of restoration) (Capellesso *et al*. 2016; Capellesso *et al*. 2018). The third area is a monoculture forest of *Eucalyptus grandis* W. Hill ex Maiden (EM) 14 years old, with 19.7 ha and a density of 665 ind ha^{-1}. The fourth area comprises a 24-year-old monoculture forest of *Pinus elliottii* Engelm. (PM), with 15 ha and a density of 415 ind ha^{-1}.

Species richness

Field inventories of all four forest types were conducted in August 2013. At each forest area, ten plots (each of 10 × 20 m) were randomly set up around 10 m apart, totaling 0.2 ha per study site. Species richness in native forest areas (NF1 and NF2) was evaluated by sampling adult tree individuals (diameter at breast height, DBH ≥ 5 cm) in each plot. The species present in different areas are listed in Table S1. The monocultures (EM and PM) had only adult trees of *Pinus elliottii* and *Eucalyptus grandis*, respectively.

C and N analysis in soil and litter

Soil subsamples were collected in the 0–5 cm layer at five points within each plot (at the four vertices and in the center of the plots) and homogenized into a composite sample for each plot, totaling 40 samples. The soils in native areas are more basic and have lower clay content compared to monoculture areas. Soil chemical characterization is available as supplementary material (Table S2). The soil was air-dried, and sieved < 2 mm.

Litter produced in the four study areas was collected monthly using 1 m long × 1 m wide × 0.15 m deep wood collectors furnished with a fine nylon mesh screen 1 mm thick. A total of 10 collectors were randomly distributed in each study area. The collected material was oven-dried at 50 ºC until constant mass, and the dry matter was determined. The annual leaf litter production (Mg ha^{-1}) in each area was evaluated monthly for 12 months (January to December 2013). Leaf litter samples were ground into a fine powder and analyzed for carbon and nitrogen contents in a Fisher Scientific Flash 2000 analyzer to calculate the litter C:N ratio.

Samples of soil and litter were further ground to < 250 μm in an agate mortar to determine organic C and total N content by dry combustion (FlashEA 1112, Thermo Electron

Corporation, Milan, Italy). The C: N ratio for soil and litter was calculated from

these values. The C and N contents in the litter were calculated considering the C and N concentration and their respective dry matter. Soil C and N stocks were calculated using the equivalent soil mass approach (Ellert & Bettany 1995), taking the soil mass of NF as a reference. The soil bulk density was determined for the 0−0.5 cm layer in six samples, using a volumetric

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0.10 m height metallic ring in the center of the soil layer at each sampling location (Blake & Hartge 1986).

$$\text{SOC stock} = \text{organic carbon} * \text{BD} * (\text{Dref}/\text{Ds}) * 10 \qquad \text{Equation 1}$$

Where SOC stock is the soil organic carbon stock (Mg ha^{-1}), organic carbon is the organic carbon concentration (g kg^{-1} soil), BD is the bulk density of the soil (g cm^{-3}), Dref is the soil density of the reference area NF1, and Ds is soil density (g cm^{-3}). Bulk density was determined with volumetric metal rings as described by Pauletto (1997). This method is used to assess SOC on a normalized soil mass per unit area basis to account for differences in soil masses caused by soil management (Gifford & Roderick 2003). The soil used as the reference was sampled from the native forest (NF1) because this area has a longer regeneration time than NF2.

Litter lignin content

The litter lignin content was evaluated according to Sausen et al. (2014). Samples of the leaf litter fraction (0.05 g) were homogenized in 50 mM potassium phosphate buffer (1.2 ml, pH 7.0) with a mortar and pestle and transferred to an Eppendorf tube (Ferrarese et al. 2000). The pellet was centrifuged (1400 ×g, 4 min) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer pH 7.0 (1.2 ml); three times with phosphate buffer pH 7.0 containing 1% (v/v) Triton® X-100 1% (v/v) (1.2 mL); twice with phosphate buffer pH 7.0 containing 1 M NaCl (1.2 ml); twice with distilled water (1.2 ml); and twice with acetone (800 µl). The pellet was dried in an oven (60 °C, 24 h) and cooled in a vacuum desiccator. The dry matter was defined as the protein-free cell wall fraction. The reaction mixture (200 µl of thioglycolic acid plus 1 ml of 2 M HCl) was then added to the Eppendorf tube and heated (95 °C, 4 h). After cooling at room temperature, the sample was centrifuged (1400 ×g, 5 min) and the supernatant was discarded. The pellet containing the lignin-thioglycolic acid (LTGA) complex was washed three times with distilled water (1.2 ml), and the LTGA was extracted by shaking (30 °C, 18 h, 160 oscillations/min) in 0.5 M NaOH (1 ml). The supernatant was stored after

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centrifugation (1400 ×g, 5 min). The pellet was rewashed with 0.5 M NaOH (500 µl) and mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (300 µl). After precipitation (0°C, 4 h), the LTGA was recovered by centrifugation (1,400 ×g, 10 min) and washed twice with distilled water (7 ml). The pellet was dried at 60 °C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm. Litter lignin content was expressed as µg of LGTA per mg of the analyzed sample.

Data analysis

The differences in species richness, leaf litter production, litter C:N ratio, lignin content, and SOC among the four areas NF1, NF2, EM, and PM were evaluated by a one-way analysis of variance (ANOVA), using an "aov" function, followed by a Tukey test, using a "TukeyHSD" function, both of the "vegan" package (Oksanen *et al*. 2022).

To evaluate the effects of species richness, litter amount, litter C:N ratio, and lignin content in SOC, a generalized linear mixed model (GLMM) was conducted, followed by a multimodel information inference approach (Burnham *et al*. 2011). This approach using GLMMs makes it possible to assess which factors tested or which set of factors (predictor variables) has the greatest influence on the response variable (SOC stocks). First, we controlled the effects of spatial autocorrelation by including sample size and areas in the model as random covariates and standardized the variables with the "scale" command from the "vegan" package (Oksanen *et al*. 2022). We calculated Akaike's information criterion of second order for small samples (AICc). The calculated average models with ΔAICc ≤ 4 were considered, and we obtained the importance value for each predictor variable from the sum of the Akaike weights (Burnham *et al*. 2011). We thought the binomial negative family, using the "glmer.nb" command for the "lme4" package. We used the "muMIn" package (Bartón 2020) in R statistical environmental (R Core Team 2022).

In addition, a principal component analysis (PCA) was performed to visualize and evaluate the effect of forest areas on the full suite of richness, soil, and litter variables determined and to

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comprehend their associations better, using a "PCA" function from "FactoMineR" package (Husson *et al*. 2023). The PCA was followed by a permutational multivariate analysis of variance (PERMANOVA) using Euclidean distance to test the ordination significance, using the "adonis2" command and the package "vegan" (Oksanen *et al*. 2022). We generated the figures using the ggplot2 (Wickham *et al*. 2023) for boxplots and "factoextra" for PCA (Kassambara & Mundt 2020) packages. All analyses were performed in the Rstudio software (R Core Team 2022).

RESULTS

Differences in native x plantations forests

The species richness was higher in NF1 than in other areas ($F_{34,3} = 225$, $p < 0.0001$; Figure 1a). The SOC was higher in NF1 than in other areas ($F_{20,3} = 32.52$, $p = 0.009$; Figure 1b).

Litter production was higher in natural areas (NF1 and NF2) than in monoculture (EM and PM) ($F_{34,3} = 49.11$, $p < 0.0001$; Figure 1c) and there were no differences between EM and PM. The litter C:N ratio was higher in both monoculture areas ($F_{34,3} = 142.2$, $p < 0.0001$; Figure 1d) compared to natural areas. Litter lignin content was higher in NF1 and EM, while the NF2 did not differ from both and PM ($F_{28,3} = 49.11$, $p = 0.009$; Figure 1e).

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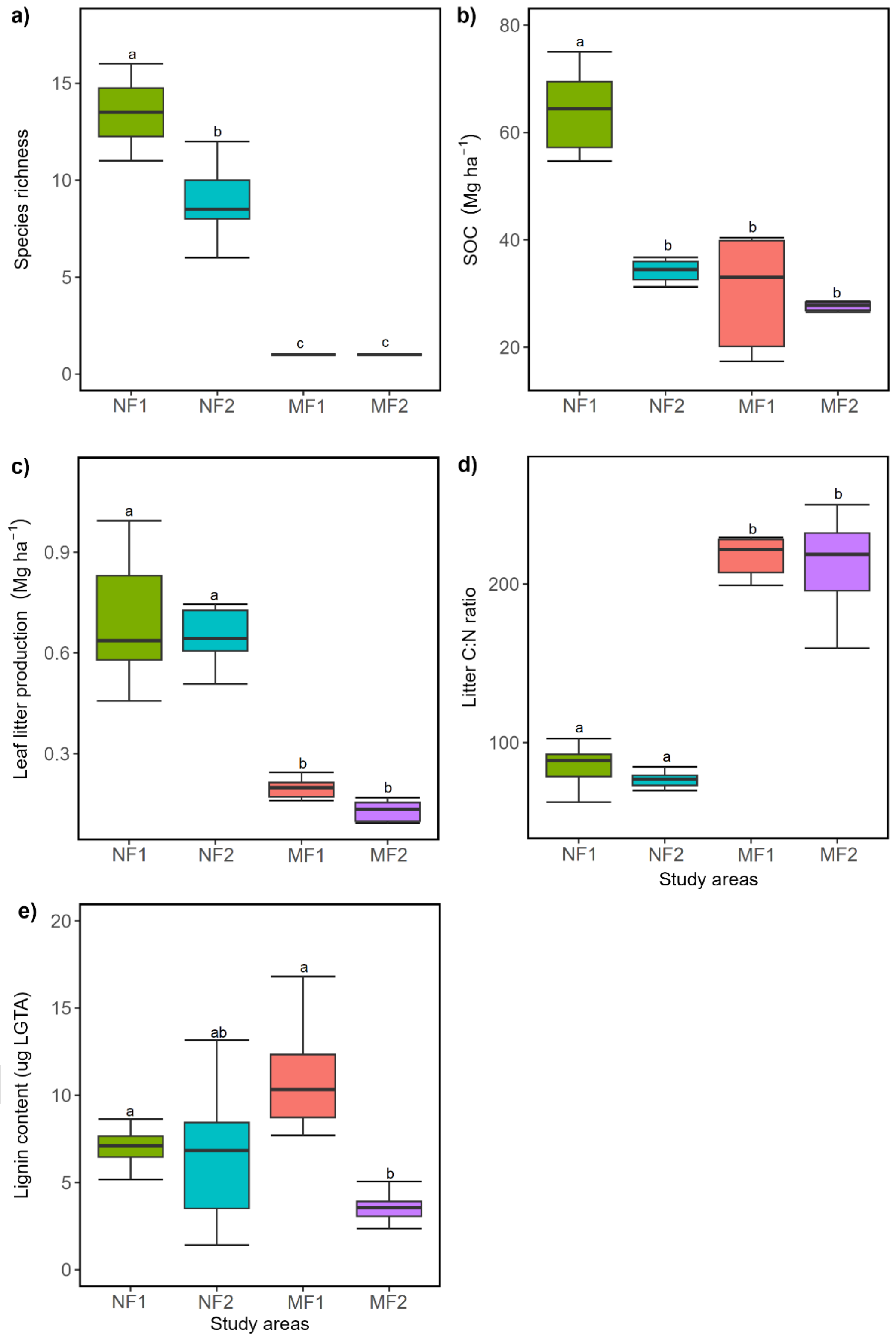

Figure 1. Differences in a) species richness, b) SOC stock, c) leaf litter production, d) litter C:N ratio, and e) lignin content among natural forest (NF1 and NF2) and monocultures of *Eucalyptus grandis* (EM) and *Pinus elliottii* (PM) forests in Southern Brazil.

SOC is associated with forest structure and litter quality

The forest age (z=2.28, p=0.02) and richness (z=2.60, p=0.009) positively influence the SOC stock, while the leaf litter C:N ratio has a negative effect on SOC (z=2.21, p=0.03) (Figure 2; Table 1). The litter production (z=0.30, p=0.76) and leaf litter lignin content (z=1.20, p=0.23) do not affect the SOC stock (Figure 2; Table 1; Table S3).

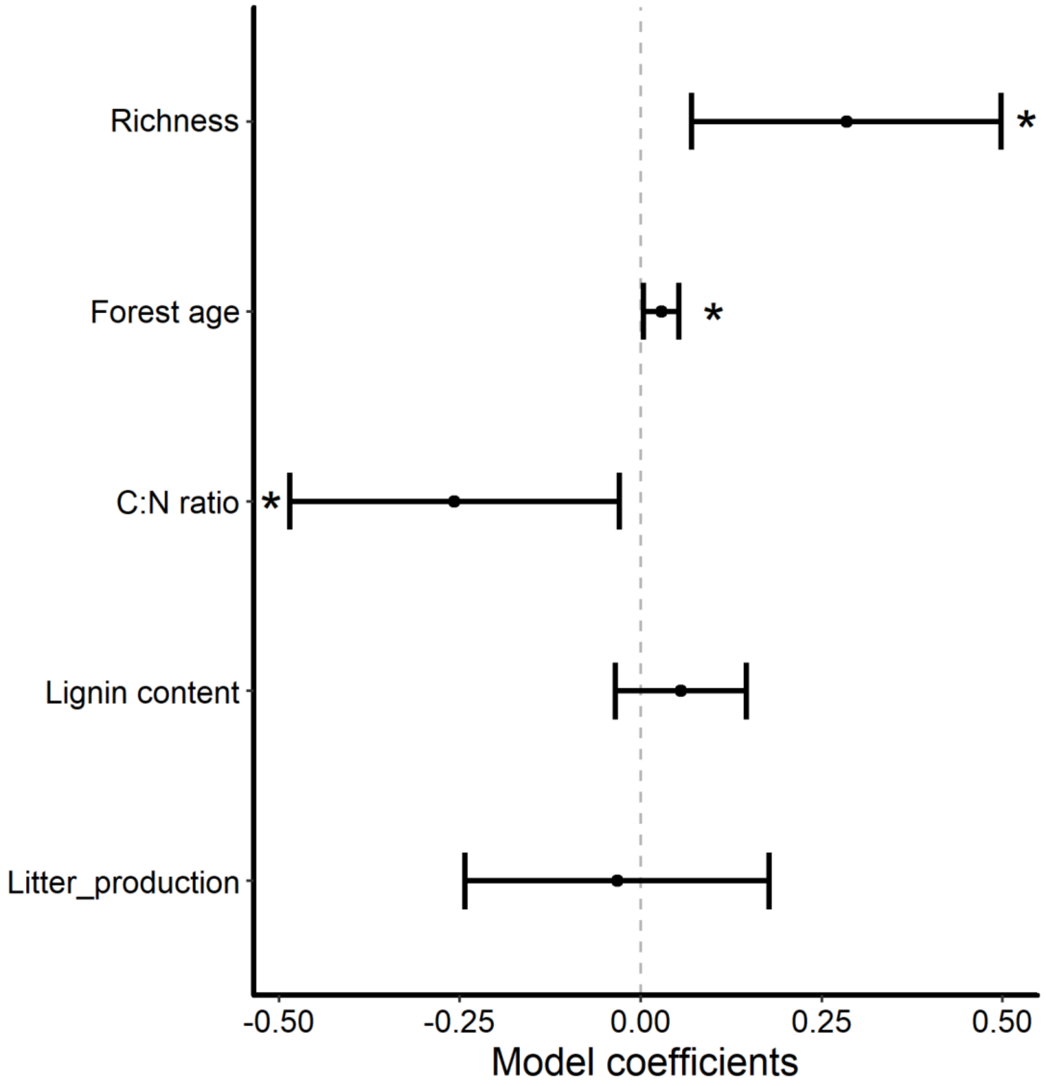

Figure 2. Effects of predictor variables (richness, forest age, litter C:N ratio, and lignin content) on the SOC stock. The positive or negative position of the bars represents an effect of the predictive variable on SOC stock. * Represent the significant effect ($p < 0.05$). The bars represent the adjusted standard errors obtained after the average model analysis.

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Table 1. Effects of forest age, richness, litter input, litter C:N ratio, and litter lignin content on SOC stock. The average model analysis was performed considering all models with values of ΔAICc ≤ 4.

	Estimate	**Std. Error**	**SE**	**Z**	**P**
Intercept	3.31	0.33	0.33	9.98	<0.0001
Forest age	0.03	0.01	0.01	2.28	0.02
Species Richness	0.28	0.10	0.10	2.60	0.01
Litter input	-0.03	0.10	0.11	0.30	0.76
C:N ratio	-0.26	0.11	0.11	2.21	0.03
Lignin content	0.05	0.04	0.05	1.20	0.23

The ordination of richness, forest age, and litter variables with PCA showed the effect and the segregation of the four forest areas, with PC1 presenting 57.7% of explanation and PC2 22.1% of explanation (Figure 3a). PC1 and PC2 accounted for 79.8% of the total variation. Further, PC1 corresponded mostly to the variation in C:N ratio and species richness,

while PC2 corresponded to differences in forest age, with the older areas (NF1 and MP) segregated from the younger ones (NF2 and EM; Figure 3b). The significance of the ordination was proven by the PERMANOVA ($F_{23,3}$ = 33.92, $p < 0.0001$, R^2= 0. 48; Table S4). The PCA results emphasize the effect of richness, litter quality, and time of natural regeneration/planting on SOC stock.

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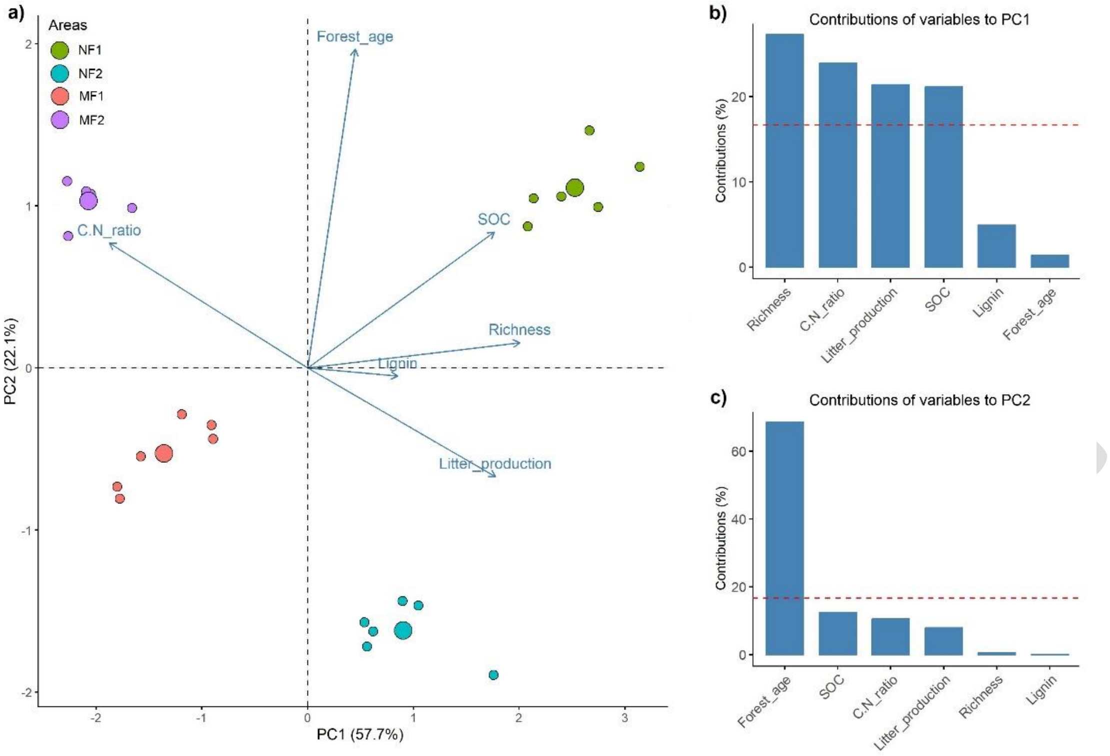

Figure 3. Principal component analysis (PCA) of soil organic carbon stock, richness, forest age, and litter quality attributes in natural forests (NF1 and NF2) and monocultures of *Eucalyptus grandis* (EM) and *Pinus elliottii* (PM). Forest age, species richness, C:N = litter C:N ratio; SOC = soil organic carbon stock; Litter = litter production; Lignin = lignin content.

DISCUSSION

In this study, we investigated which factors drive the soil carbon accumulation in native subtropical Brazilian Atlantic Forest and monocultures (with non-native species) to explain the differences in soil carbon storage between them. We found the positive effect of species richness, forest age, and litter C:N ratio on SOC stocks. The higher SOC stock can be associated with the floristic structure of natural forests (richness and regeneration time).

Recent studies in subtropical forests also observed a positive effect of tree richness on SOC storage (Liu *et al*. 2018, Li *et al*. 2019, Li *et al*. 2020, Xu *et al*. 2020). However, these studies addressing how diversity drives soil carbon accumulation were conducted in areas with low richness (3-20 tree species) (Liu *et al*. 2018, Li *et al*. 2019, Wang *et al*. 2022) or in plantations with multiple species (Wang *et al*. 2020). Our results highlight the effects of plant taxonomic diversity on SOC storage from a small fragment approach, showing that natural regeneration and increased plant diversity can be considered as a strategy for forest carbon management (Liu *et al*. 2018), even in small forest fragments, which are characteristic of subtropical Atlantic Forest ecosystems.

A meta-analysis on carbon stock and diversity in restored Brazilian biomes showed that diversity and carbon levels tend to increase with restoration age (Jakovac *et al*. 2024). Another interesting finding from this study is that most restoration initiatives analyzed are less than 20 years old. In naturally regenerating secondary forests, it takes more than 20 years for carbon and biodiversity to reach levels like the original systems in forest biomes (Poorter *et al*. 2016, Rozendaal *et al*. 2019). Our results reinforce the importance of natural regeneration for the recovery of SOC stocks. Young forests (20-30 years old) may be important for the carbon pool, as an increase in diversity is expected over time.

SOC storage is determined by the balance between carbon input (litter) and output (litter decomposition and existing SOC), and diversity has the potential to affect all these processes, determining the soil C balance. Some studies associate higher carbon stock with litter quality (lower C:N ratio) (Li *et al*. 2020, Teixeira *et al*. 2023). However, richness can directly increase SOC storage by mechanisms other than litter quality. In the mixed-species planted forest, changes in SOC stocks could result from a trade-off between leaf litter and fine root carbon inputs (Wang *et al*. 2022). According to these authors, mixing 4-5 tree species can lead to a higher SOC level than having fewer or more tree species in a planted subtropical forest.

Furthermore, the PCA analysis evidenced the segregation among the forest areas according to richness, forest age, litter production, and C:N ratio. Our results showed that differences in SOC stock between natural forest and monocultures were not associated with litter quantity and lignin, but natural forest areas had a lower C:N litter compared to monocultures. Teixeira *et al*. (2023) observed that areas with higher quality organic residues (lower C:N ratio) boost the storage of C and N in the soil, reinforcing our results.

Although the analyses conducted in this study indicate the positive effect of richness and forest age on SOC, we emphasize two points. First, due to the small number of forest fragments

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located in the study area, our study presents a limitation regarding the number of evaluated fragments. Second, the analyses conducted sought to minimize the effect of size and isolated the spatial autocorrelation between the areas. Thus, our results indicate a positive effect of richness on SOC stock, suggesting a co-benefit between the time of natural regeneration and richness. Nevertheless, we recommend that future studies with a larger number of areas and regeneration times be conducted to explore the effects of plant diversity on carbon storage capacity in forest fragments undergoing natural regeneration.

In subtropical landscapes, the abandonment of agricultural areas involves two main land cover conversion processes for environmental compensation: planting non-native tree monocultures or natural regeneration. Although tree planting is considered an important strategy for mitigating climate change and carbon sequestration, our results infer that higher richness in the subtropical Brazilian Atlantic Forest drives SOC stocks. The subtropical forest fragments comprise young areas that are in natural regeneration (<30 years of passive restoration) (Rovani *et al*. 2019), and the native forests evaluated in this study showed 53 and 33 species, which is higher than the richness of other studies in subtropical forests analyzing how diversity drives soil carbon storage (Liu *et al*. 2018; Li *et al*. 2019; Wang *et al*. 2022). Thus, we emphasize that natural regeneration brings benefits compared to tree monoculture even in highly fragmented landscapes, as observed in Southern Brazil. Additionally, studies conducted in a chronosequence of natural regeneration are important to verify co-benefits between richness and regeneration time on soil carbon stock.

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Supplementary Material

Table S1. Species list in naturally restored subtropical forests (NF1 and NF2) in Southern Brazil.

Table S2. Chemical characteristics, clay, and pH of the soil, under the native areas (NF1 and NF2) and of *Eucalyptus grandis* (EM) and *Pinus elliottii* (PM) forests, with 0-5 cm depth. Mean ± standard deviation. N= 6. Different letters show the differences between areas.

Table S3. Selection of the best models using the Akaike information criterion (AIC). All models with $\Delta \leq 4$. AICwi = Akaike information criterion weight.

Table S4. PERMANOVA results from the ordination of forest age, species richness, C:N ratio, Litter amount, litter production and SOC variables.

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