Soil Respiration and Organic Carbon Changes along a Chronosequence of *Pinus nigra* Forest Stands

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Understanding the trajectory of changes in soil respiration (R_s) and soil organic carbon (SOC) with stand ages of the black pine (*Pinus nigra* Arnold) forest is essential for forest management and carbon budget estimates. In this research, changes of R_s and SOC were studied with respect to stand age in a chronosequence of three age classes of *P. nigra* plantations consisting of young (0 to 10-year-olds), middle-aged (11- to 20-year-olds), and pre-mature (35- to 45-year-olds) forest stands. R_s rates, soil temperature, and soil moisture were measured using an automated dynamic survey chamber (Li-8100A) for a year, encompassing summer, fall, winter, and spring seasons. Mean R_s significantly increased from young- to middle-aged and then stabilized, with effluxes ranging from 2.46 to 2.94 µmol CO_2 m^{-2} s^{-1}. Forest litter significantly increased with stand age, but not the SOC in the mineral soil layers. The R_s showed a positive correlation with soil temperature (0.77) and air temperature (0.75) but not with soil moisture (-0.43). The present results highlight the importance of stand age in assessing carbon budget and provide essential information for forest managers and stakeholders in evaluating the potential of *P. nigra* forests as tools for carbon sequestration and mitigating global warming impacts.

DOI: 10.15376/biores.19.3.6095-6119

Keywords: Stand age; CO_2 emissions; Soil temperature; Soil moisture; Black pine; Dynamic chamber

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INTRODUCTION

Under the Kyoto Protocol of the UNFCC, carbon sequestration in the afforestation and reforestation projects is one of the prescribed options to enhance the removal of atmospheric greenhouse gases (IPCC 2001). Article 12.5 of this Protocol requires that the certified emission reductions under the clean development mechanism (CDM) must be “environmentally additional” to what would have occurred without the project activities based on the tangible and measurable quantity of GHGs relative to the baseline (Baumert 2000). For this reason, it is essential to quantify the baseline GHG emissions accurately to prevent “over-crediting,” which could be done by estimating and validating the associated emissions and removals of GHGs (Pearson *et al*. 2013). Although there is no rigid rule for carbon sequestration accounting, the carbon budget method, which includes measuring the C input and output fluxes to and from the ecosystem, is considered the most accurate method of accounting for C sinks (Saint Andre *et al*. 2007).

Quantifying carbon budgets of a given forest ecosystem requires quantification of the different components of the C cycle, including the C inputs in the above- and below-ground biomass and soil organic carbon (SOC), as well as the fraction of gross primary production that is lost through plant respiration (Trenberth 2005; Zscheischler *et al*. 2014; Berg and Sheffield 2018; Xu *et al*. 2021). Plant respiration, the main pathway through which a large quantity of CO_2 returns into the atmosphere, comprises soil respiration (roots and soil organisms) and respiration from the aboveground biomass (foliage, stems, and branches) (Wang *et al*. 2023; Pacaldo *et al*. 2024). Soil respiration (R_s) constitutes a large proportion of ecosystem respiration, contributing about 80% to 90% of the total plant respiration rates (Raich and Schlesinger 1992; Schimel *et al.* 2001). Some authors estimated that the CO_2 emissions from the R_s are about 78 to 95 Pg of CO_2 emissions back into the atmosphere annually (Bond-Lamberty and Thomson 2010; Hashimoto *et al*. 2015) and are considered the largest source of CO_2 emissions (IPCC 2021; Nissan *et al*. 2023). Because of the large amount of CO_2 emissions from R_s and SOC stocks (Raich and Schlesinger 1992; Schimel *et al*. 2001; Nissan *et al*. 2023) any small changes in these components of the C cycle could dramatically alter the C budget of a given ecosystem (Pacaldo *et al*. 2024).

The stand age of the forest plantation may exert a strong influence on R_s and SOC because as the forest develops and ages, some structural, morphological, and physiological changes occur, which are likely to affect the C cycling and other vital processes in the forest ecosystem (Yu *et al.* 2014). Many researchers have invested considerable efforts into understanding the age effects of forest ecosystems on R_s and SOC. However, these studies suggest different trajectories of stand age effects on R_s rates: (1) increased (Peichl *et al*. 2010; Peichl *et al*. 2014; Song *et al*. 2017; Yu *et al.* 2019); (2) decreased (Darenova *et al*. 2016); (3) no change (Chang *et al*. 2020); (4) decreased and then increased (Payeur *et al*. 2012); (5) increased and then stabilized (Uri *et al*. 2022); (6) and nonlinear response with high variability (Smith *et al*. 2010; Kukumägi *et al*. 2017). Similarly, the trajectory of SOC changes is less certain and may vary with stand age and other factors (Chen *et al.* 2010). Reported chronosequence studies conducted in forest ecosystems revealed inconsistent findings on stand age effects on the SOC: (1) increases with stand age (Li *et al*. 2013; Cheng *et al*. 2014; Zhao *et al*. 2014; Francis-Justine *et al*. 2015; Song *et al.* 2017; Smal *et al.* 2019; Lei *et al.* 2019; Chen *et al.* 2020; Zhu *et al.* 2020; De Marco *et al*. 2021); (2) decreases with stand age (Cao *et al.* 2012; Amir *et al*. 2018; Chen *et al.* 2010;); (3) nonlinear response, *i.e.* a decrease and then increase with stand age (Covington 1981; Chen *et al*. 2013; Pacaldo *et al.* 2013), and (4) no significant pattern (Noh *et al*. 2010; Huang *et al*. 2021). The inconsistency of SOC changes with stand age may partly be explained by the complicated dynamics in the accumulation and decomposition of soil organic matter (Chen *et al.* 2013).

However, in the literature and to our knowledge, there has been no investigation conducted that compares and assesses the changes in R_s rates and SOC with stand ages of *Pinus nigra* stands. The deficiency of data precluded our understanding of the carbon sequestration potentials of different developmental stages of *P. nigra* stands, which is a widely distributed tree species in Turkiye with an estimated area of about 4.2 million hectares (Sakici *et al.* 2018; Pacaldo *et al*. 2024). Determining the trajectory of R_s and SOC changes with stand age is essential for accurate C budget estimates in forest ecosystems. Whether the R_s rates and SOC values significantly change as the forest ages is a critical question this study seeks to investigate. In this investigation, it was hypothesized that the R_s rates and SOC would increase with stand age. The objectives of this study were (1) to assess the R_s and SOC changes with stand age and (2) to determine the environmental factors affecting R_s. Quantifying the magnitude and trajectory of R_s and SOC changes with stand age provides valuable information to advance our understanding of the dynamics of the carbon budget in the *P. nigra* ecosystem, which is a critical factor in achieving accurate estimates of abated anthropogenic CO_2 emissions by the reforestation and afforestation projects under the CDM of the Kyoto Protocol (Baumert 2000).

EXPERIMENTAL

Study Site

A field experiment was conducted in a black pine (*Pinus nigra* Arnold) forest at Kastamonu City, Türkiye, which is geographically located between 41°22'19.89 "N and 33°44'4.10"E, with a mid-latitude temperate climate under the Köppen classification system (Turkes 2020), and an annual temperature mean of 10 °C, and annual precipitation mean of 538 mm (Turkish State Meteorological Service 2024). The forest plantation was established simultaneously, but restocking, replanting, and natural regeneration in canopy gaps resulted in the variability of age classes, in which the exact age was determined using an increment borer. The management history, soil properties, topography, and silvicultural treatments of the research site were more or less similar; hence, it is safe to assume homogeneity of the R_s and SOC before establishing the plantation forests. Based on the World Reference Base (WRB) classification system, the site's soil is dominantly Lithic Leptosol (Özden *et al*. 2001; Aksoy *et al*. 2010), overlying calcareous and sedimentary rocks in the advanced stage of weathering. Soil properties of the study site are summarized in Table 1. The study site is a reforestation area containing a continuous track of homogenous and well-managed *P. nigra* plantations (35- to 45-year-olds) with considerable forest gaps in the periphery and inside the forest stands where the naturally regenerated forests of different age classes (5- to 10-year-olds and 11- to 15-year-olds) can be found. A timber inventory was carried out to determine tree stocking and sizes, which are summarized in Table 2.

Experimental Design

A field experiment in a complete randomized block design (10 m × 10 m) was established across regeneration and plantation forest stands. Three age classes of *P. nigra* plantations and one control were selected in this study. Age classes were based on the age of stands during the 2023 growing season. These included young forest stands (0- to 10-year-olds), middle-aged stands (11- to 20-year-olds), pre-mature stands (35- to 45-year-olds), and control (treeless undisturbed sites located along forest borders). Each of the three age classes, including the control, was replicated four times, represented by 16 sampling plots (*i.e.*, three age classes + control × 4 replications) distributed across four blocks.

In this experiment, the following parameters were evaluated: R_s, SOC, soil temperature, air temperature, soil moisture, and stand age. The key parameters include the R_s and SOC, which are the main focus of the investigation. These parameters constitute the main components of output and input in the C cycle and C budget estimates; hence, any small changes in these components can dramatically alter the C balance of the ecosystem. Soil temperature, air temperature, and soil moisture affect R_s rates and soil organic matter decomposition.

Soil Respiration Measurement

An automated soil CO_2 efflux measurement system (LI- 8100A) equipped with a 10-cm survey chamber (LI-8100-103), soil temperature probe (6000-09TC Omega), and

EC-5 soil moisture sensor (Li-COR, NE, USA) was used to measure the R_s, soil temperature, air temperature, and soil moisture. The analyzer unit (LI-8100) houses the infrared gas analyzer (IRGA) and stores the data. The survey chamber is equipped with a pressure vent on its top, alleviating the errors due to differences in pressures between inside the chamber and the ambient environment (Liang *et al*. 2004; Xu *et al.* 2006). The base of the chamber is fitted with a rubber seal, which prevents air leakage in and outside the chamber's headspace during measurements. During measurements, the chamber moves automatically into the soil collar by the control of the analyzer unit, which pumps air into the chamber. Measurement of R_s rates was done by mounting the survey chamber on the top of the soil collar with a total duration of 240 seconds for each measurement, consisting of 30 seconds of equilibration/deadband (*i.e*., length of time when chamber closes completely and mixes with air before measurement begins), 150 seconds observation length, and 60 seconds of purge time (Pacaldo *et al.* 2024). Simultaneous measurements were made of soil temperature and soil volumetric moisture content with the R_s at 5 cm soil depth.

In each plot, one polyvinyl chloride (PVC) soil collar (5 cm diameter and 7 cm height) was inserted into 4 cm soil depth, leaving a 3 cm soil collar. The soil collars were inserted a few days before the first measurement to minimize the effects of soil disturbance and artifacts. The R_s was measured for one year, encompassing the four seasons (summer, fall, winter, and spring). Bi-monthly R_s measurements were conducted, except during the winter months (January to March), wherein a monthly measurement was done. In winter, the snowpacks were removed carefully from the soil collars before mounting the survey chamber on the top of the soil collars. The R_s between 10:00 and 16:00 were measured based on previous studies using continuous and unattended R_s measurements in the field (Pacaldo *et al*. 2014). The annual cumulative R_s was evaluated using the mean values and scaled to metric tons of CO_2 per hectare per year (Mt CO_2 $ha^{-1}yr^{-1}$) using a conversion factor 12.59 (Pacaldo *et al*. 2024). Calculations were done also for the sensitivity of R_s to soil temperature using the Lloyd and Taylor (1994) model based on an exponential relationship,

$$R_s = R_{ref}\,\exp^{E_0 T} \quad (1)$$

$$Q_{10} = \exp^{10E_0} \quad (2)$$

where R_{ref} (µmol m^{-2} s^{-1}) is the basal respiration at the reference temperature, E_0 (°C) is the parameter of temperature sensitivity, T (°C) is the soil temperature, and Q_{10} refers to temperature sensitivity, representing the response of R_s to a 10 °C increase in temperature (Han *et al*. 2023).

Soil and Forest Litter Sampling

Composite soil samples were collected at two 20 cm increment depths: 0 to 20 cm as topsoil and 21 to 40 cm as subsoil. A metal cylindrical bulk density corer (5 cm dia. x 5 cm ht) was used to collect soil samples in the field. Before collecting soil samples, organic matter (undecomposed and partially decomposed) was removed to the depth of the mineral soil surface. Then, the bulk density soil corer was hammered into the middle section of each soil layer depth.

Composite forest litter and other organic debris on the soil surface (O horizon) were collected in each plot using a forest litter sampler (20 cm dia. × 20 cm ht). The samples were transported to the laboratory and weighed per plot to determine the total green (fresh).

The dry matter weight was determined randomly by oven-drying the samples at 105 °C to constant weight.

Soil Laboratory Analysis

We measured the soil pH and electrical conductivity (EC) using a Hanna HI 9812-5 pH/EC/TDS/Temperature meter in a 1:2.5 soil-water mixture, and EC was then converted to EC of saturated paste extract using the equation ECe = 4.34 × EC1:2.5 - 0.17 (Sonmez *et al*. 2008) for sandy soils in Türkiye. We determined the soil organic carbon content using the Loss-On-Ignition method by burning the soil samples at 450 °C for 12 hours. The values of SOM were converted to SOC using a conversion factor of 1.72 (Post *et al*. 1982; Nelson and Sommers 1983). We used an established procedure for the hydrometer method (Bouyoucos 1962; Gangwar and Baskar 2019) to determine the different proportions of soil particles (clay, silt, and sand). We used a textural triangle to determine the textural classification of the soil.

The physical and chemical analyses revealed that the site's soils, both upper and subsurface layers, are moderately dense, with values ranging from 1.47 to 1.75 g cm^{-3} and 1.42 to 1.89 g cm^{-3}, respectively. The soil reactions (pH) are nearly neutral, ranging from 7.53 to 7.65 in the upper 10 cm depth and 7.60 to 7.75 in the subsurface layer. The soil EC is slightly saline, ranging from 3.2 to 4.72 dSm^{-1} in the upper 10 cm depth and 2.85 to 3.63 dSm^{-1} in the subsurface layer. Generally, the site has a sandy loam soil texture (Table 1).

Table 1. Soil pH, EC, BD, and Soil Texture at the Upper and Lower Layers of Different Forest Types (Mean ± MSE; *n* = 4)

Soil Depth	Age Class (yr-old)	pH	EC$_e$ (dSm^{-1})	BD (g cm^{-3})	Soil Texture		
					Sand (%)	Clay (%)	Silt (%)
0 -10 cm	0 to 10	7.65 ± 0.06	3.20 ± 0.61	1.64 ± 0.28	70	21	9
	11 to 20	7.53 ± 0.05	4.25 ± 0.46	1.54 ± 0.16	70	21	10
	35 to 45	7.58 ± 0.02	4.72 ± 0.42	1.47 ± 0.09	71	23	6
	Control	7.55 ± 0.03	3.68 ± 0.24	1.75 ± 0.13	64	24	12
	p-value	0.27	0.14	0.70	0.30	0.44	0.12
11-20 cm	0 to 10	7.75 ± 0.03	2.85 ± 0.32	1.89 ± 0.28	68	23	9
	11 to 20	7.65 ± 0.09	3.10 ± 0.45	1.60 ± 0.07	61	24	15
	35 to 45	7.60 ± 0.04	3.63 ± 0.04	1.42 ± 0.09	73	21	6
	Control	7.60 ± 0.04	3.43 ± 0.24	1.73 ± 0.36	66	24	10
	p-value	0.21	0.32	0.56	0.17	0.91	0.02

Aboveground Biomass Inventory

A tree inventory was carried out to determine the tree density and estimate the size of aboveground biomass. In each stand age, four 20 m x 20 m sampling plots were delineated for a total sampling area of 800 m^2 or eight percent (8%) sampling intensity per hectare. In each sampling plot, the diameter breast height (DBH) and height of all trees within the plot's borders were measured. A steel increment borer was used to determine the tree's exact age. The biomass was estimated based on the formula CAG =0.010dbh^{2}h, developed by Sakici *et al.* (2018) for *P. nigra* in the study site. The results of the inventory are summarized below (Table 2).

Table 2. Characteristics of the Aboveground Standing Biomass of *P. nigra* Forest in the study site (Mean ± MSE)

Stand Age (year-old)	Tree Density (Trees ha^{1})	Mean DBH (cm)	Mean Height (m)	Total Biomass Aboveground (Mg ha^{-1})*
0-10	4300.00	1.4 ± 0.12	0.97 ± 0.01	0.23
11-20	3900.00	5.0 ± 0.29	4.39 ± 0.18	13.61
35-45	438.00	24.0±0.77	12.83 ± 0.35	65.94
Control	0.00	0.00	0.00	0.00

*Calculated using the equation: Biomass = 0.020 DBH^2 x Height (Sakici *et al*. 2018)

Statistical Analyses

The R_s rates of different forest types and controls were analyzed using the general linear (PROC GLM) model, in which treatment was considered as the main effect, block as the random effect, and time as a second qualitative factor to test if the R_s vary among different measurement times. The significant differences in R_s and SOC among treatments were tested using one-way analysis of variance (ANOVA) with $p < 0.05$ considered a significant value. Multiple-wise comparisons were done with Tukey's test to separate significant differences among treatment means. Pearson correlation analysis determined the relationship between soil respiration, soil temperature, soil moisture, and air temperature. The relationships among soil respiration, soil temperature, air temperature, and soil moisture were analyzed using regression analyses based on collected data throughout the study. Values of r-square, Mallows' C_p Statistics, Akaike Information Criterion (AIC), and mean standard error (MSE) were used to select the number of independent variables in the multiple regression model (Pacaldo *et al*. 2024). All statistical analyses were performed using a SAS Statistical Package (SAS 9.1 SAS Institute).

RESULTS

Soil Respiration Rates

Soil respiration rates significantly differed across all sources of variation. The R_s showed highly significant differences among treatment (age classes) ($p = 0.009$) and time ($p < 0.0001$). However, the combined effects of time and treatment on R_s rates did not show significant differences ($p = 0.089$) (Table 3).

Table 3. Results of ANOVA Test for R_s Rates Among Treatments, Time, and Interaction Effects Between Treatment and Time ($n = 240$; $p = 0.05$)

Source of Variation	df	MS	F- value	P- value
Treatment	3	3.72	5.76	0.0009
Time	11	41.17	63.81	<0.0001
Time × Treatment	24	0.42	0.66	0.089

Mean separation using Tukey's test revealed that the middle-aged stand (11- to 20-year-olds) exhibited the highest R_s rates, which is significantly different from young stand (0- to 10-year-olds) and the control, but not with the R_s of the 35-45-year-olds (pre-mature) plantation. The R_s rates, ranging from 2.36 to 2.94 µmol m^{-2} s^{-1}, agreed well with some reported values in literature (*e.g.,* Wiseman and Seiler 2004; Payeur-Poirier *et al.* 2012; Luan *et al*. 2012; Pang *et al*. 2013; Wei *et al*. 2022; Amarille *et al*. 2023; Pacaldo and Aydın 2023; Tong *et al*. 2023). The cumulative annual R_s rates ranged from 29.72 to 37.06 Mt CO_2 ha^{-1} yr^{-1} (Table 4).

Table 4. Mean Annual R_s and Cumulative Annual R_s Rates of All Age Classes and Control

Stand Age (Year-old)	Mean Annual Soil Respiration Rates (µmol CO_2 m^{-2} s^{-1})	Cumulative Annual Soil Respiration (Mt CO_2 ha^{-1} yr^{-1})
0-10	2.36 (0.19)^b	29.72 (2.44)
11-20	2.94 (0.20)^a	37.06 (2.53)
35-45	2.60 (0.21)^{ab}	32.76 (2.59)
Control	2.46 (0.21)^b	30.97 (2.84)

*Values with the same letters are not significantly different at a 95% confidence level, based on Tukey's Test

Seasonal Soil Respiration Rates

In the winter season, the R_s rates showed no significant differences in all stand ages, including the control. The middle-aged stand in springtime demonstrated significantly higher R_s than other treatments. From summer to fall periods, the control indicated the highest R_s. However, it did not significantly differ from the middle-aged and pre-mature stands (Fig. 1). Proportional seasonal contributions to the total cumulative annual soil CO_2 emissions showed that winter contributed only about 8.84 %. In comparison, summer contributed about twice as much (42.60%) as spring (21.66%) and fall (26.90%) (Fig. 2), which is consistent with some previous reports (*e.g.,* Groffman *et al*. 2001; Liptzin *et al*. 2009; Pacaldo *et al*. 2012, 2024).

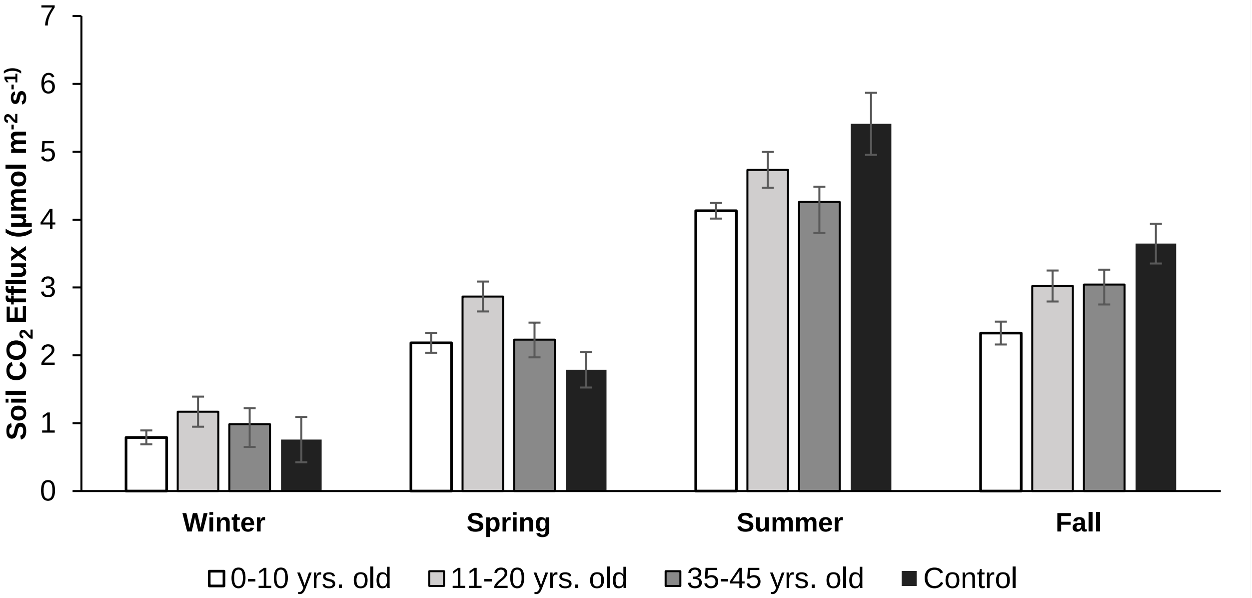

Fig. 1. Mean seasonal R_s rates in all age classes and control (n = 16; Mean ± MSE)

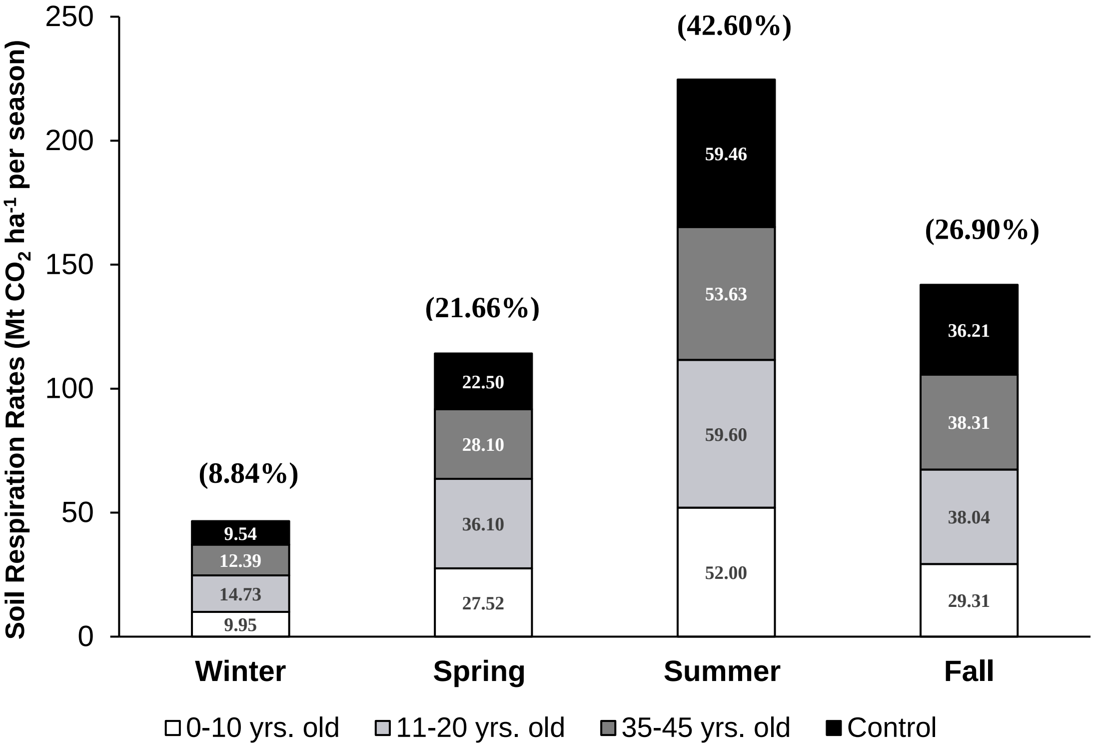

Fig 2. Proportion of soil respiration (R_s) in all stand ages and control (Mt CO$_2$ ha^{-1} per season) and seasonal contribution of R_s to the cumulative annual CO$_2$ emission rates (%)

Relationship between Soil Respiration, Temperature, and Soil Moisture

Pearson's correlation analysis showed a strong significant relationship between R_s and soil temperature (0.77) and air temperature (0.75), indicating that it tends to increase with increasing soil and air temperatures. In contrast, the R_s showed a weak negative significant relationship with soil moisture (-0.43), suggesting that the R_s tend to decrease with increasing soil moisture contents. Notably, the soil temperature and air temperature showed a robust positive correlation (0.91), suggesting that the air temperature provides a good approximation of the soil temperature (Pacaldo *et al.* 2024). The soil moisture demonstrated a stronger negative relationship with the air temperature (-0.58) than with soil temperature (-0.45), indicating that the soil moisture tends to dry faster with an increasing air temperature than with soil temperature (Table 5). The R_s significantly increased in dryer than saturated soil conditions (Figs. 3 and 4). The sensitivity analysis showed higher sensitivity of R_s to temperature below 20 °C, with sensitivity Q_{10} values ranging from 2.8 to 7.3. The sensitivity of R_s with soil temperature more or less stabilizes with increasing temperature above 30 °C, with Q_{10} values ranging from 1.28 to 1.39.

Table 5. Estimated Pearson's Correlation Coefficients of the Relationship between Soil Respiration, Soil Temperature, Air Temperature, and Soil Moisture (*n* = 240)

	R_s	Soil T	SM	AirT
R_s		0.77; (p<0.001)	-0.43; (p<0.001)	0.75; (p<0.001)
SoilT	0.77; (p<0.001)		-0.45; (p<0.001)	0.91; (p<0.001)
SM	-0.43; (p<0.001)	-0.45;(p<0.001)		-0.58; (p<0.001)
AirT	0.75 (p<0.001)	0.91; (p<0.001)	-0.58; (p<0.001)	

Abbreviations: R_s, soil respiration; SoilT, soil temperature; AirT, air temperature; SM, soil moisture

The highest R_s rates were observed in the summer months (*i.e.*, June to August), with soil and air temperatures ranging from 17.47 to 22.81 °C and soil moisture from 18.4% to 18.5%. The trend follows a gradual decrease in R_s rates at the start of wet months in September to the end of winter in February. Following the melting of snow, gradual increase in soil and air temperatures, and increased soil moisture in spring (*i.e.*, March through May), the R_s rates gradually increased until they peaked in June. The R_s showed significant positive relationships with soil temperature and air temperature (0.56) but indicated a negative relation with soil moisture (Figs. 3 and 4). These findings suggest that soil respiration increases with an increase in soil and air temperatures, while it decreases as soil moisture increases.

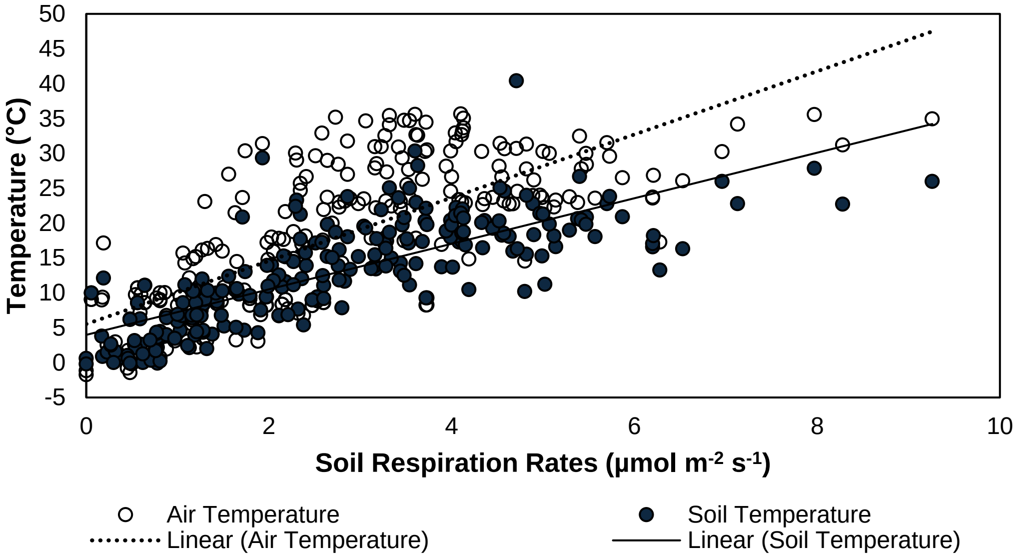

Fig 3. Soil respiration rates as a function of soil and air temperatures. The correlations show a significant strong positive relationship of R_s rates with soil (0.77) and air temperature (0.75) (*n* = 240).

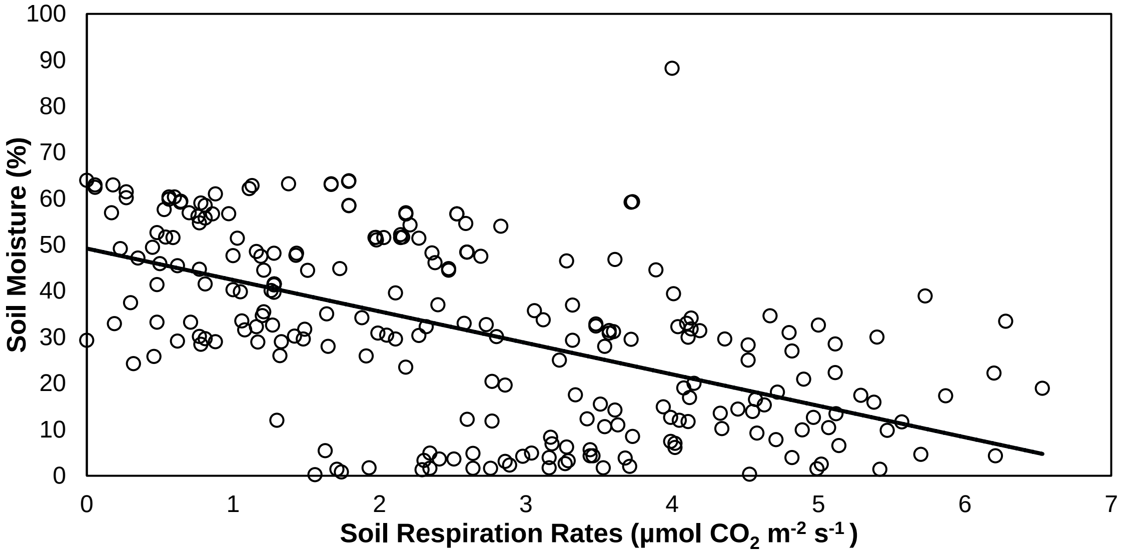

Fig. 4. Soil respiration rates as a function of soil moisture. The correlation shows a weak negative relationship of *R*s with soil moisture (*n* = 240).

The calculated values were R^2, Mallow's C_p statistics, Akaike Information Criterion (AIC), and mean standard error (MSE) to determine the variables useful for predicting R_s rates. Variables with the highest R^2 but low values of Mallow's C_p, AIC, and MSE are considered as best candidate variables for the multiple regression model (Pacaldo *et al*. 2024). Table 6 summarizes the analysis results, which showed that the soil temperature and soil moisture provide a good approximation of R_s, as indicated by a high R^2 value but low C_p, AIC, and MSE. Including air temperature in the model did not significantly improve the precision of the model in predicting R_s. Estimated parameter estimates of the regression line of the multiple regression model are summarized in Table 7, which shows that the intercept was not significantly different from zero ($p = 0.0898$). The soil temperature is shown to be the only variable with a significant probability value ($p < 0.0001$), suggesting that the soil temperature is a significant parameter in predicting R_s rates.

Table 6. Estimated r^2 values, Mallows' C_p Statistics, Akaike Information Criterion (AIC), and Mean Standard Error (MSE)

Number in Model	R-square	Cp	AIC	MSE	Variables in model
1	0.63	2.78	-0.59	0.99	SoilT
1	0.56	41.53	34.91	1.19	AirT
1	0.13	261.39	163.91	2.32	SM
2	0.64	2.00	-1.41	0.98	SoilT SM
2	0.63	4.17	0.79	0.99	SoilT AirT
2	0.56	43.47	36.86	1.19	SM AirT
3	0.64	4.00	0.59	0.98	SoilT AirT SM

Abbreviations: SoilT, Soil temperature: AirT, Air temperature; SM, Soil moisture.

Table 7. Estimated Line Intercepts and Constants Values for Independent Variables in the Multiple Regression Model

Variables	Parameter Estimates	Standard Error	*p*-value
Intercept	0.4385	0.3574	0.0898
Soil temperature	0.1393	0.0247	<0.0001
Air temperature	0.0317	0.0188	0.0930
Soil moisture	-0.0029	0.0046	0.5252

Changes in Soil Organic Carbon

The ANOVA revealed no significant differences in SOC contents among age classes and interaction effects (Table 8). By contrast, the SOC significantly differed between soil depths ($p = 0.0002$), with the upper 15-cm soil depth containing significantly lower SOC contents in the upper 10 cm (18.60 to 25.68 Mt C ha^{-1}) than the subsurface layer (31.31 to 45.30 Mt CO$_2$ ha^{-1}) of the mineral soil. The soil organic matter contents on the soil surface (O-horizon) significantly differed in all stand ages, with increasing accumulation of SOM as the forest ages (11.14 to 78.57 Mt SOM ha^{-1}) (Table 9). As expected, the control contained the smallest volume of litter because it receives SOM inputs from grasses only.

Table 8. ANOVA Showing Differences in SOC among Stand Age, Soil Depths (0 to 10 cm and 11 to 20 cm), and the Interaction Effects between Age and Soil Depth ($n = 4$; p = 0.05)

Source	DF	MS	F- value	P- value
Age	3	78.24	0.64	0.60
Depth	1	2300.12	18.76	0.0002
Age*Depth	3	447.33	1.22	0.32

Table 9. Soil Organic Carbon of the Study Sites' Different Soil Layers and Forest Litter Deposits

Soil Depth	Age classes (yr-old)	BD (kg m^{-3})	SOM (%)	SOC (Mt C ha^{-1})	Forest Litter (Mt SOM ha^{-1})
0 to 10 cm	0 to 10	1.64 ± 0.28	2.23 ± 0.60	18.60 ± 2.84	11.14 ± 1.93^{c}
	11 to 20	1.54 ± 0.16	3.01 ± 0.73	25.68 ± 5.13	34.00 ± 1.29^{b}
	35 to 45	1.47 ± 0.09	2.31 ± 0.47	20.33 ± 5.12	78.57 ± 3.78^{a}
	Control	1.75 ± 0.13	1.81 ± 0.30	18.78 ± 3.91	4.42 ± 0.61^{d}
	p-value	0.70	0.491	0.640	
11 to 20 cm	0 to 10	1.89 ± 0.28	1.53 ± 0.40	31.31 ± 6.31	
	11 to 20	1.60 ± 0.07	1.81 ± 0.30	32.94 ± 4.43	
	35 to 45	1.42 ± 0.09	2.49 ± 0.39	41.58 ± 7.96	
	Control	1.73 ± 0.36	2.45 ± 0.53	45.39 ± 6.81	
	p-value	0.56	0.306	0.939	

Subscripts in the same letters denote non-significant relationships between forest types of the same soil layer at a 95% confidence level based on Tukey's test (Mean ± MSE; $n = 4$).

DISCUSSION

Stand Age Influence on Soil Respiration Rates

The chronosequence approach provides a means to investigate processes that may take decades to develop by utilizing sites of different ages as treatment or basis for describing patterns attributable to individual sites as they age (Yanai *et al*. 2000). In this study, the R_s increases from young (0- to 10-year-olds) to middle-aged (11- to 20-year-olds) stands and then stabilizes onwards, as indicated by the non-significant differences of R_s rates between middle-aged and pre-mature (35- to 45- year-olds) stands (Table 4). At a young age, R_s increases because of high tree density per square meter, rapid growth, and high fine root production (Litton *et al*. 2003; Montagnoli *et al*. 2012; Pregitzer *et al*. 2000), until it reaches the stability period, which usually occurs at the middle-aged when the canopy fully occupies the available space (Helmisaari and Hallbacken 1999; Vanninen and Makela 1999; Makkonen and Helmisaari 2001; Børja *et al*. 2008; Tang *et al*. 2009; Claus and George 2011; Konôpka *et al*. 2011). When the forest matures, the root and shoot growth ratio stabilizes and reaches an equilibrium in which there is a balance between ecosystem production (inputs) and ecosystem respiration (outputs) (Van Noordwijk and De Willigen 1987; Vogt *et al*. 1987). The present finding agrees well with some previous studies, reporting R_s increases at a young age (Makkonen and Helmisaari 2001; Sulzman *et al*. 2005; Sayer *et al*. 2007; Prévost-Bouré *et al*. 2010; Zhuang *et al*. 2023), peaks at middle ages, and stabilizes at the time of canopy closure (Law *et al*. 2003; Bond- Lamberty *et al.* 2004; Wiseman and Seiler 2004; Saurette *et al*. 2006; Tang *et al*. 2009; Arevalo *et al*. 2010; Chang *et al*. 2020).

However, some studies found lower or higher R_s values due to stand density, canopy gaps, root biomass, forest litter production, soil organic matter inputs, and other environmental factors (Irvine and Law 2002; Mayer *et al*. 2017; Chin *et al*. 2023). Varik *et al.* (2015) reported an increasing trend of R_s from young to middle-aged and then stabilized to pre-maturity age due to equilibrium between SOC input and heterotrophic respiration at the maturity period. Similarly, Peichl *et al.* (2010) observed an increased R_s with stand age in the *Pinus strobus* forest in Canada due to a steady increase of SOM from aboveground biomass and roots. Other authors also reported a similar pattern in the loblolly pine forest in Virginia, U.S.A (Wiseman *et al.* 2004), boreal jack pine forest in Canada (Smith *et al.* 2010), and Scots pine in Estonia (Uri *et al.* 2022). Payeur-Poirier *et al.* (2012) observed a decreasing trend of R_s after harvest and then increased with further stand development. In the Scots pine forest in Canada, Uri *et al.* (2022) reported that R_s showed similar patterns following seasonal fluctuations in soil temperature irrespective of stand age. Other authors reported no change in R_s with stand age in the hybrid poplar plantations in Canada (Chang *et al.* 2020), Norway Spruce forest in Estonia (Kukumägi *et al.* 2017), and White pine (*Pinus strobus*) in Canada (Peichl *et al.* 2014).

Surprisingly, the control showed significantly higher R_s than the 0 to 10 and 35 to 45 age classes in the summer (Fig. 1), suggesting that treeless areas could contribute a higher CO_2 into the atmosphere during warmer periods. The high CO_2 emission rates in treeless areas covered by grasses (control) could probably be explained not only by the direct exposure of the soil surface to solar radiation and high temperature but also by the influence of litter quality (Han *et al*. 2015; Petraglia *et al*. 2019). Grasses produce a highly decomposable organic matter, hence a rich source of labile carbon, which drives microbial activities and R_s rates (Post and Kwon 2000; Saurette *et al*. 2006; Teklay and Chang 2008; Petraglia *et al*. 2019; Chang *et al*. 2020; Pacaldo *et al*. 2024). In contrast, the R_s rates under pine forests did not dramatically increase during summer, probably due to the high acid-nonhydrolyzable residue (AUR) or lignin contents of needles, bark, and other residues of pines that inhibit decomposition rates and microbial activities (Prescott 2010; Hasbullah and Marschner 2015).

Environmental Factors Affecting Soil Respiration

In this study, the R_s showed a strong positive correlation with soil temperature (0.77) and air temperatures (0.75), indicating the tendency of R_s to increase with temperatures, which agrees well with some previous reports with the strength of the relationship ranging from moderate to a strong relationship (Dinca *et al*. 2018; Cui *et al*. 2020; Pacaldo *et al*. 2023, 2024; Amarille *et al*. 2023; Pacaldo *et al*. 2024). The R_s rates increase as the temperature rises because autotrophic and heterotrophic activities respond positively to high temperatures, particularly at times of abundant soil moisture contents, which could drive decomposition rates (Salah and Scholes 2011; Petraglia *et al*. 2019). This pattern conforms with our calculations on the sensitivity of R_s to temperature, in which we found that R_s is highly sensitive to increasing temperature with Q_{10} values ranging from 2.8 to 7.3 at temperatures ranging from 5 °C to 19 °C, consistent with some previous reports (Han *et al*. 2023).

In contrast, the R_s was negatively correlated with soil moisture, indicating an inversely proportional relationship pattern (Amarille *et al*. 2023; Pacaldo *et al*. 2023; Pacaldo *et al*. 2024). However, findings on the relationship between R_s and soil moisture are not consistent, with some authors reporting a positive relationship (Raich and Schlesinger 1992; Wood *et al*. 2013; Fei *et al*. 2015), negative relationship (Adachi *et al*. 2005; Yanni *et al*. 2020), and no significant relationship (Borken *et al*. 2006; Bréchet *et al*. 2009). Sealing of soil pores, which occurs when soil pores are thoroughly saturated with water, and freezing effects of cold temperature during winter results in suppression of R_s due to the reduced diffusion of CO_2 and decreased microbial and root activities (Du *et al*. 2013; Chang *et al*. 2014; Pacaldo *et al*. 2024). In this study, it was also observed that the R_s significantly decreased when the soil moisture dropped to 5.74%, despite the warm temperature (>15 °C), suggesting that, during dry periods, the soil moisture mainly regulates R_s, not the temperature, particularly at times when the soil water becomes the limiting factor of microbial and root activities.

In this study, a combination of soil temperature and moisture factors could strongly predict R_s rates, as indicated by high r-square but low values of Mallow's C_p, AIC, and MSE, suggesting that these factors influence each other and R_s rates (Dinca *et al*. 2018; Pacaldo *et al*. 2024), probably due to their direct influence on root and microbial activities (Subke and Bahn 2010; Chang *et al*. 2014), ecosystem productivity, and hydrological processes (Kanmani et al. 2023).

Stand Age Effects on Soil Organic Carbon

Contrary to the hypothesis, the statistical analysis failed to detect significant differences in SOC with stand age. The lack of statistically detectable significant changes in the upper 10 cm of the mineral soil suggests that changes in SOC in the pine forest ecosystem occur at a prolonged process, and these changes could not be statistically detected not only due to high spatial variations but also because of slow transformation rate of forest litter into soil organic carbon. Recalcitrant organic matter, such as roots, usually decays very slowly, which can be detected only after a few years (Prescott 2010; Hasbullah and Marschner 2015; Aydin *et al*. 2018). In contrast, the significant differences in forest litter on the soil surface across different age classes demonstrate that the decomposition rate of organic matter from *P. nigra* occurs at a very slow pace resulting in its steady accumulation on the forest floor as the forest ages (Table 6). Lignin (AUR), abundant in the forest litter of pines, provides an effective shield against a rapid decomposition process and inhibits microbial activities (Prescott 2010).

Reported chronosequence studies in other forest ecosystem types suggested different trajectories of SOC changes with stand age. A study in the northern hardwood, New Hampshire, predicted a 50 % loss of organic matter in the first 20 years before the disturbed site slowly recovered in the next 50 years and then stabilized onwards. The author attributed the decreased SOM to the rapid loss of organic matter in young stands due to increased decomposition and reduced litter inputs (Covington 1981). Chen *et al.* (2013) reported a similar pattern of SOC with stand ages in the Chinese fir (*Cunninghamia lanceolate*), due to management regimes, climate, and edaphic conditions interacting with the SOC. In Pakistan, a chronosequence of Chir Pine (*Pinus roxburghii*) revealed a decreasing trend of SOC with stand age due to disturbances of forest management operations (Amir *et al*. 2018, 2019). Chen *et al.* (2010) reported a similar pattern for the Mongolian pine (*Pinus sylvestris* var. *mongolica* Litv.) in China, wherein the SOC decreased from 12 to 40 years due to disturbance caused by wind erosion.

In contrast, Smal *et al*. (2019) reported an increasing SOC with stand age because litter production exceeded decomposition, gradually increasing SOC in the organic layer. De Marco *et al*. (2021) observed a similar pattern in Stone pine (*Pinus pinea*) in Italy, wherein the SOC increases with stand age with about 80 % accumulation in the organic horizon due to high litter production. Other authors also reported a similar pattern of increased SOC with stand age in Chinese pine (*Pinus tabulaeformis*) (Zhao *et al.* 2014), lacebark pine (Li *et al*. 2013), Mongolian pine (*Pinus sylvestris var. mongolica*) (Lei et al. 2019), *Pinus massoniana* (Song *et al*. 2017), and boreal larch forest (*Larix gmelinii*) (Zhu *et al.* 2020). Although Yanai *et al.* (2000) found no pattern in the change in organic matter with time in the Northern Hardwood, New Hampshire, they observed the highest organic matter accumulation in oldest stands and the least in young and middle-aged stands.

Surprisingly, the subsurface layer (11 to 20 cm depth) contained significantly higher SOC stocks compared to the upper 10 cm depth, which is somewhat inconsistent with the authors’ expectations and findings in some previous reports (Smal *et al*. 2008; Bayramin *et al*. 2009; Pacaldo 2012; Pacaldo *et al*. 2013). The higher SOC contents in the subsurface may be associated with the cultural treatment received by the soil before the reforestation project. The plowing and disking of the soil, part of the site preparation, resulted in mixing the soil organic matter in the plow layer (Ap) into the subsurface layer, which remained in place and protected against the attack of decomposers. In contrast, the decomposition of the SOM in the upper 10 cm is expected to occur much faster than the subsurface layer because it is directly exposed to ambient conditions and is the optimum depth of rapid microbial and root activities (Wang *et al*. 2019; Hao *et al*. 2021).

CONCLUSIONS

The present findings failed to support the hypothesis. The R_s rates in the *P. nigra* forest ecosystem significantly increased from the young (0 to 10-year-olds) to middle-aged (11- to 20-year-olds) stands and then stabilized onwards. In contrast, the SOC in the mineral soil showed no statistically significant differences at two 10-cm soil depth increments, with higher SOC contents in the subsurface layer than the upper 10-cm depth. Forest litter on the soil surface significantly increased with stand age and accumulated in large quantities as the forest ages. Although these results may not be conclusive to other types of forest ecosystems, these findings provide additional information regarding the trajectory of R_s and SOC changes in the forest ecosystem with stand age. To our knowledge, this is the first chronosequence study assessing the R_s and SOC changes for the *P. nigra* forest, a vital piece of information in the assessment of the carbon budget in this type of forest ecosystem. The present findings could also be used in alleviating the uncertainty regarding the critical question of whether to "freeze" the reference baseline over the project's lifetime in the accounting of carbon sequestration of registered CDM reforestation projects, particularly for *P. nigra* forest. The results also imply that periodic recalculation of the baseline, *e.g.*, every five years, is needed to increase the reliability and accuracy of the "baseline" estimates, as highlighted by significant increases of R_s rates from young to middle-aged stands and continued accumulation of forest litter as the forest ages. The SOC contents in the mineral soil layers are C neutral, indicating that this C pool could be frozen over the forest's lifetime, at least in the *P. nigra* plantations.

Although this study increased our understanding of the stand age effects of SOC and R_s rates in the *P. nigra* forests, there is no absolute certainty whether the observed trajectory is due to changes of age or other factors because of the high spatial variation of soil organic matter stocks, roots, and microclimate conditions. A chronosequence approach is subject to several potential sources of error due to site variability and other environmental factors affecting R_s and SOC that could be unrelated to the time since the establishment of the forest or the occurrence of disturbance (Yanai *et al.* 2000). Furthermore, it is also beyond the scope of this study to assess the microclimate effects on R_s and SOC due to differences of canopy sizes and tree height or trees within the borders of different stand age classes. Moreover, climate change impacts and other environmental stresses would also create uncertainties about this observed trajectory of R_s and SOC changes in the *P. nigra* forest, particularly in the event of forest fires. These subjects are interesting topics for future investigations, particularly in light of changing climate patterns.

ACKNOWLEDGMENTS

The authors are deeply grateful to Prof. Dr. Renato S. Pacaldo of the Mindanao State University-Marawi City and a visiting professor at the Kastamonu for his guidance in preparing the manuscript and conducting this study. Grateful appreciation also goes to Randell Keith Amarille, a graduate student of Kastamonu University, for his invaluable assistance in data collection and analysis, laboratory analysis, and manuscript preparation.

FUNDING SUPPORT

This study was supported by the Turkey Scientific and Technological Research Council (Türkiye Bilimsel ve Teknolojik Araştırma Kurumu) (TÜBİTAK), the Science and Fellowship Grant No. 121C066, under the CoCirculation2 with funding from the European Union's Horizon 2020 research and innovation program under the Marie Sktodowska-Curie grant agreement No. 801509.

DECLARATION OF COMPETING INTEREST

As part of our ethical obligation as a researcher, we declare that no competing interest is involved in this study, and the research outcome, either financial, commercial, or non-financial conflicts.

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Article submitted: April 19, 2024; Peer review completed: June 16, 2024; Revised version received: July 7, 2024; Accepted: July 8, 2024; Published: July 18, 2024.
DOI: 10.15376/biores.19.3.6095-6119