New Phytologist

Research

Fine root production in a chronosequence of mature reforested mangroves

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Received: *22 January 2021*
Accepted: *28 April 2021*

New Phytologist (2021) **232:** 1591–1602
doi: 10.1111/nph.17480

Key words: belowground carbon, blue carbon, coastal wetland, EnRoot minirhizotron, fine root production, reforestation, restoration, root trait.

Summary

• Mangroves are among the world's most carbon-dense ecosystems, but have suffered extensive deforestation, prompting reforestation projects. The effects of mangrove reforestation on belowground carbon dynamics are poorly understood. In particular, we do not know how fine root production develops following mangrove reforestation, despite fine root production being a major carbon sink and an important control of mangrove soil accretion.

• Using minirhizotrons, we investigated fine root production and its depth variation along a chronosequence of mature Vietnamese mangroves.

• Our results showed that fine root production decreases strongly with stand age in the uppermost 32 cm of our soil profiles. In younger mangrove stands, fine root production declines with depth, possibly due to a vertical gradient in soil nutrient availability; while root production in the oldest stand is low at all depths and exhibits no clear vertical pattern. A major fraction of fine root production occurs deeper than 30 cm, depths that are commonly omitted from calculations of mangrove carbon budgets.

• Younger mangroves may accrue shallow soil organic matter faster than older mangroves. Therefore, root productivity and forest stand age should be accounted for when forecasting mangrove carbon budgets and resistance to sea-level rise.

Introduction

Background and rationale

Mangroves provide ecosystem services, such as flood protection and carbon sequestration, that have been estimated to be worth US$194 000 per hectare per yr (Costanza *et al.*, 2014, using data from 2011); however, these valuable coastal wetlands are being lost rapidly (Duke *et al.*, 2007; Richards & Friess, 2016). In response, reforestation projects have emerged worldwide (Lee *et al.*, 2019). Mangrove reforestation has intensified since it was recognised that mangroves are among the world's most carbon-dense ecosystems and protect the coast from storms (Bosire *et al.*, 2008; Donato *et al.*, 2011; Lee *et al.*, 2014). Compared with the aboveground domain, belowground carbon dynamics remain poorly understood and are not commonly monitored following mangrove reforestation. However, up to 90% of mangrove ecosystem carbon is stored in the soil, of which fine roots are one of the primary sources, along with riverine sediments (Middleton & McKee, 2001; Bouillon *et al.*, 2008; McKee, 2011; Ezcurra *et al.*, 2016). As such, the resistance of reforested mangroves to sea-level rise, and the long-term trajectory of the carbon store in reforested mangroves, are unclear.

Mangroves have been shown to resist sea-level rise by accreting soil through increased soil organic matter (SOM) accumulation (Middleton & McKee, 2001; McKee, 2011; Krauss *et al.*, 2014). In doing so, they have been described as occupying vertical 'accommodation space' (Rogers *et al.*, 2019). SOM accumulation results from root production and other sources (e.g. litterfall, algae, tidal OM deposition) exceeding SOM decay (Middleton & McKee, 2001; McKee, 2011; Ezcurra *et al.*, 2016). Several studies have indicated that SOM decay does not decrease with sea-level rise in mangroves and saltmarshes (Blum, 1993; Kirwan *et al.*, 2013; Arnaud *et al.*, 2020), meaning that mangroves require enhanced root production or sedimentation to accrete and persist in the face of sea-level rise. However, root production is one of the least studied components of the carbon cycle in mangroves, and little is known about its controlling factors, especially following reforestation (McKee & Faulkner, 2000; Bouillon *et al.*, 2008; Alongi, 2009; Perez-Ceballos *et al.*, 2018; Muhammad-Nor *et al.*, 2019). A few studies have investigated the response of root production to nutrients, flooding, and their interactions (Naidoo, 2009; Castañeda-Moya *et al.*, 2011; Adame *et al.*, 2014; Cormier *et al.*, 2015; Poungparn *et al.*, 2016; Torres *et al.*, 2019).

The effects of mangrove reforestation on root production have received little attention in the published literature, despite a proliferation of mangrove reforestation projects in recent years (e.g. in Bangladesh, Philippines and Senegal) (Lee *et al.*, 2019). To our knowledge only two studies have investigated the impacts of reforestation on root production, and they showed contrasting results. McKee & Faulkner (2000) found that reforested mangroves had similar belowground productivity to natural mangroves. They investigated two different locations, where mangroves have been reforested. The reforested mangroves at their study sites were 9 and 19 yr old, and both were compared with adjacent natural mangroves. By contrast, Perez-Ceballos *et al.* (2018) showed that three reforested mangroves (2, 3 and 5 yr old, with different inundation regimes and mangrove genera) were much less productive than a natural mangrove. The effect of reforestation on belowground carbon production is also likely to change as a replanted mangrove stand ages (Fromard *et al.*, 1998; Walcker *et al.*, 2018), yet, to date, no study has investigated the temporal development of belowground carbon production following mangrove reforestation. Aside from decades-long monitoring programmes, a chronosequence approach allows the effects of stand age upon belowground carbon cycling to be investigated. However, finding a chronosequence of mature reforested mangroves with comparable environmental conditions is challenging.

The depth distribution of mangrove root production has been little studied (Castañeda-Moya *et al.*, 2011; Muhammad-Nor *et al.*, 2019), but is likely to be important in regulating carbon input into the soil, resource acquisition by mangrove trees and rhizodeposition (e.g. release of labile organic compounds by roots in the rhizosphere) (Kuzyakov *et al.*, 2000; Johnson *et al.*, 2001; Iversen *et al.*, 2012; Sokol & Bradford, 2019; Sokol *et al.*, 2019). Production of both coarse and fine roots has long been believed to be concentrated in the upper 30 cm of the soil, with little or no production below this depth. Only three mangrove studies have investigated root production in detail below 30 cm (Castañeda-Moya *et al.*, 2011; Xiong *et al.*, 2017; Muhammad-Nor *et al.*, 2019). These studies suggest that production deeper than 30 cm may be an important fraction of the total. However, the factors controlling this depth distribution are poorly understood (Castañeda-Moya *et al.*, 2011; Muhammad-Nor *et al.*, 2019), and it is currently unknown how reforestation might affect the depth distribution of mangrove root production. This knowledge is important for estimating the quantity and quality of carbon stored in reforested mangroves, as a major source of slow-cycling organic carbon in soils is thought to derive from fine roots (Rasse *et al.*, 2005; Sokol & Bradford, 2019; Sokol *et al.*, 2019; Kida & Fujitake, 2020). McKee *et al.* (2007) and Xiong *et al.* (2017) both showed that the accumulation of fine roots (which they defined as having diameter < 2.5 mm and < 1 mm, respectively) was a major contributor of SOM accumulation in mangroves, and correlated significantly with soil surface elevation in mangroves. In addition, with the rise of temperature and atmospheric CO_2 concentrations, many ecosystems have experienced deeper root production and an increased rate of rhizodeposition, which have cascading effects on ecosystem carbon dynamics (Sadowsky & Schortemeyer, 1997; Phillips *et al.*, 2011). Recent evidence from mangroves has shown significant changes in root dynamics due to warming (Coldren *et al.*, 2019).

The *in situ* measurement of fine root growth is operationally challenging, because it is difficult to access the root system frequently without disturbing the soil. Unlike other root production measurement techniques, minirhizotrons allow repeated observations of the same roots or soil space to be made over extended periods, with limited soil disturbance after an initial settling-in period. Minirhizotrons also have the advantage of distinguishing root production from the simultaneous decay of root detritus, while avoiding artefacts from artificial or disturbed soil substrates that can affect root-ingrowth bags. We are not aware of any previous studies that have reported the use of minirhizotrons in mangroves, which may be due in part to their being poorly suited to wetland conditions, and their high costs. However, a recently developed minirhizotron system, EnRoot, is tailored to mangrove conditions, enabling the growth of individual roots to be tracked *in situ* at multiple depths, with little disturbance (Arnaud *et al.*, 2019).

Aim and hypotheses

We used the EnRoot minirhizotron technique described by Arnaud *et al.* (2019) to investigate fine root production in one of the largest restored mangroves in the world, in the Mekong Delta in Vietnam. We used a chronosequence to test whether:

(**1**) fine root production differed with stand age in reforested mangrove;

(**2**) fine root production had depth-related patterns that accorded with reforested mangrove stand age.

Materials and Methods

Study area: restored mangroves in southern Vietnam

The Mekong Delta in southern Vietnam offers unique opportunities for investigating the trajectory of mangrove root production after reforestation. The mangroves in the Mekong Delta were extensively damaged, and in many cases completely destroyed, during the US–Vietnam war (1955–1975) through the spraying of napalm and herbicides, particularly the so-called Agent Orange (2,4,5-trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid, both with traces of dioxins) and Agent White (2,4-dichlorophenoxyacetic acid and Picloram) (Hong & San, 1993). Our study area, Cần Giờ (Fig. 1), was one of the most heavily affected areas (Hong & San, 1993), in which more than 80% of the original mangrove forest was destroyed (Oxmann *et al.*, 2010). Mangrove reforestation efforts began there more than 40 yr before our measurements and were spread over several decades, resulting in mangrove stands of different ages within the same area. The close proximity of reforested stands of different ages allowed us to investigate the impact of stand age on fine root production, while controlling for important confounding factors such as climate and tidal inundation regime (see the 'Environmental conditions at the study sites'

subsection below). The mangroves there are also likely to be representative of reforested mangroves elsewhere, as they have been planted with trees from the *Rhizophora* genus (they are, essentially, a monoculture of *Rhizophora* spp.), which is the most common genus used in mangrove reforestation worldwide (Ellison, 2000; López-Portillo *et al.*, 2017), and is the second most important mangrove genus in terms of global soil carbon stock (Atwood *et al.*, 2017). Cần Giờ is now protected under the statute of the Cần Giờ World Biosphere Reserve (UNESCO-MAB). The mangroves of Cần Giờ are nationally important because they are the only buffer between the sea and Ho Chí Minh City, the most populous city in Vietnam (Fig. 1).

We chose three sampling locations in Cần Giờ (Fig. 1) that fulfilled the following criteria: (1) the management board of Cần Giờ were able to identify confidently the date of reforestation, corroborated by historical images from Google Earth; (2) the hydrological conditions of the three sites were similar (see Section 'Environmental conditions at the study sites' below); and (3) the three sites were reforested with *Rhizophora* propagules. The sites were restored in 1978 (40 yr old at the time of our measurements), 1986 (32 yr) and 1991 (27 yr). The sites were located within 2 km of one another. At each site, we established a monitoring plot of 250 m², within which our minirhizotrons were installed at random locations. In the 27-yr-old and 40-yr-old sites, the length and width of the plots were identical (25 × 10 m). In the 32-yr-old site, the shape of the plot differed slightly (20 × 12.5 m) to minimise risks from venomous snakes and other hazards, and allowing access to an exit road in case of accidents.

Environmental conditions at the study sites

To provide contextual information about our study sites, we measured several environmental variables. The frequency of daily inundation was measured in a central point in each plot using a dipwell fitted with a self-logging water-level sensor (Solinst Levelogger Edge 3001), corrected for barometric pressure (using a Solinst Barologger Edge). Porewater temperature at each site was measured every 10 min at a depth of 1.2 m in the central dipwell using the same logger as that used for water level. We acknowledge that temperatures might have fluctuated more strongly at shallower depths, and that such fluctuations might have influenced the mangrove productivity of our stands (Ball *et al.*, 1988; Medina, 1999). Mean bulk soil electrical conductivity – a measure of soil salinity – was estimated on one occasion for the uppermost 5.5 cm of the soil profile from 20 random points in each plot using a Decagon GS3 probe (accuracy better than ± 10%; Dettmann & Bechtold, 2018) in October 2018. Average tree stem density for each plot was also measured for trees at least 1.37 m high (after Kauffman & Donato, 2012). Finally, on one occasion we measured soil nutrient (ammonium, nitrogen and phosphorus) contents in each plot at two depths (8–10 cm and 70–72 cm) in six soil cores, collected from randomly chosen locations using a gouge auger. To avoid disturbance, the cores were intentionally located away from minirhizotron tubes used for measuring fine root production (see Section 'Fine root production measurements' below). After collection, all samples were transported to the laboratory in a cool box and then frozen before analysis. We determined total soil nitrogen, phosphorus and soil ammonium (after its extraction using KCl) using a Skalar SAN++ autoanalyser. In addition to plot-specific information, we measured air temperature and relative humidity continuously using an Extech RHT10 sensor/logger at a central point between the three monitoring plots. We obtained total daily precipitation from the Can Thanh weather station (situated < 10 km from our three plots) from the Vietnamese Centre for Hydro-Meteorological Data. The environmental conditions are summarised in Table 1 and Fig. 2.

Tidal inundation frequency was the same across the sites, and soil salinity and soil temperature were similar between sites (Table 1). Tree density varied substantially, with the greatest tree

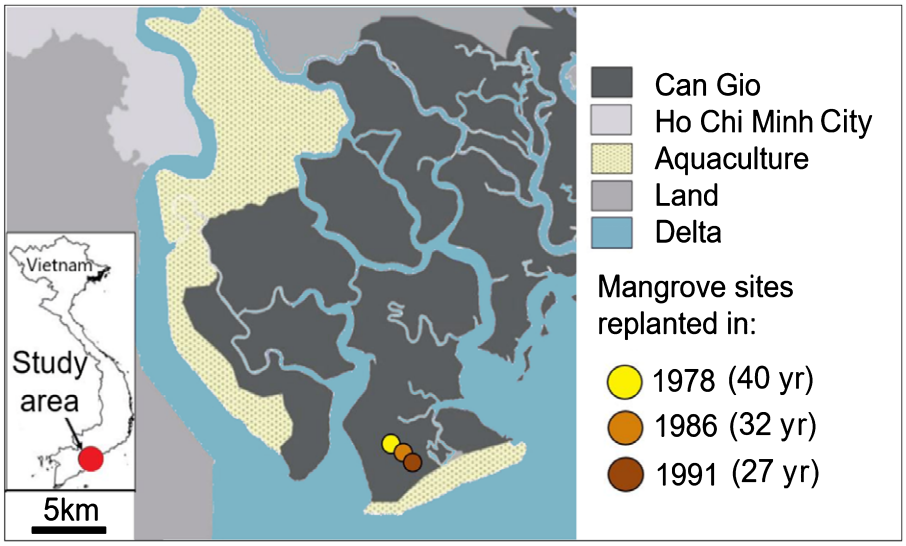

Fig. 1 Location of our study area and sites, modified from Taillardat *et al*. (2018). Sampling locations identified by year of replanting, with stand age at time of sampling in 2018 in parentheses.

density in the 27-yr-old site (0.37 trees m^{-2}), followed by the 32-yr-old (0.25 trees m^{-2}) and 40-yr-old (0.09 trees m^{-2}) sites. Mean air temperature (27.0°C) and relative humidity (91.6%) remained almost constant, but average daily precipitation was highly variable between the three measurement periods, ranging from 3.2 to 12.3 mm d^{-1}. The first measurement period (September to October) had the highest average daily rainfall. The last period (November to December) had the second-highest average, but almost 90% of the rain occurred in 1 d during tropical storm Usagi (24 November 2018).

Fig. 2 shows differences in some soil nutrient pools between sites and depths. We tested whether differences exist in soil total phosphorus and nitrogen, and soil ammonium, between the sites and between depth intervals, using linear mixed models, with site and depth as fixed effects and soil core as a random effect. For soil total nitrogen and phosphorus, we used the R function LMER from the LME4 package (Bates *et al.*, 2015). For ammonium, we used an aligned rank test (equivalent to a nonparametric linear mixed effects model), using the R function *art* from the ARTOOL package (Kay & Wobbrock, 2020), because of nonnormally distributed residuals that were not remedied by data transformation. We used the *emmeans* function from the R package EMMEANS (Lenth, 2020) to conduct Tukey post hoc comparisons between estimated marginal means for all pairs of samples.

Mean soil ammonium concentrations ranged between 0.28 (27-yr-old site) and 0.36 mg l^{-1} (32-yr-old site). These differences were marginally nonsignificant between sites of different ages ($P = 0.08$). Mean soil total nitrogen per site ranged from 2.27 (27-yr-old site) to 3.50 mg g^{-1} (32-yr-old site). Only the 32-yr-old site was significantly different from the two other sites ($P < 0.001$). The mean soil phosphorus varied from 0.17 (27-yr-old site) to 0.42 mg g^{-1} (40-yr-old site), and differed across all sites ($P < 0.01$ for all sites), with the highest value in the 40-yr-old site, followed by the 32-yr-old site, and then the 27-yr-old site.

Soil total phosphorus and nitrogen decreased significantly between the upper (8–10 cm) and lower (70–72 cm) sampling depths ($P < 0.02$ in all sites). The difference between depths was most pronounced for soil phosphorus. Mean soil ammonium concentration per site decreased across depth in the two youngest sites, but increased with depth in the 40-yr-old site. Only the decrease of the 27-yr-old site was significant ($P = 0.05$).

Table 1 Environmental conditions at the study sites.

Parameter	Stand age		
	27 yr	32 yr	40 yr
Inundation frequency per day	1.7	1.7	1.7
Soil water temperature (°C)[a] ($n = 1$ location per site)	27.5	27.8	27.1
Bulk soil electrical conductivity (mS cm^{-1})[b] ($n = 20$)	12.7	12.6	13.2
Tree density (trees m^{-2}) (whole plot average)	0.37	0.25	0.09
Air temperature (°C)[a]	27.0		
Air relative humidity (%)[a]	91.6		
Precipitation (mm d^{-1})[a]	7.7		

[a]Mean for the whole measurement period.
[b]Mean of all the readings at each site.

Fine root production measurements

We measured fine root (< 2 mm diameter) production using the EnRoot system, a minirhizotron designed for use in mangroves (see full description in Arnaud *et al.*, 2019). Commercially available minirhizotrons were unsuitable because their soil tubes were too large to fit between the stilt roots of *Rhizophora* and were impractical for remote swamps (too heavy, too large, camera not waterproof, need for power supply). Previous studies of mangrove fine root production have used root-ingrowth cores or sequential coring. There is no current comparison of methods for fine root production in mangroves. However, Hendricks *et al.* (2006) demonstrated that ingrowth cores and sequential coring are likely to underestimate fine root production, and that this underestimation was most pronounced in forest soils with high soil moisture contents. Between February and March 2018, we installed 21 EnRoot minirhizotron soil tubes at randomly selected locations across our three monitoring plots (seven minirhizotron tubes per plot) following the procedure described by Johnson *et al.* (2001) and Iversen *et al.* (2012). The location of each tube was randomised to minimise bias, and all were located far from creeks or channels. We generated seven random pairs of coordinates for each site to install our tubes (21 pairs of random coordinates in total). When a location fell within the basal area of a tree trunk, we generated another random coordinate. We did not specify a consistent distance from trees, or from prop roots, because our aim was to characterise root production at the plot level and not for individual trees. The tubes were 120 cm long and had an outside diameter of 3.2 cm. We painted the uppermost 20 cm (the part of the tube above the soil surface) to avoid light penetration. We also sealed the tops of the tubes with rubber bungs and nontoxic aquarium-grade silicone sealant to prevent water ingress between sampling (Iversen *et al.*, 2012).

We installed all the tubes vertically (90°) into preaugered holes with a slightly smaller diameter than the minirhizotron tubes. The bottoms of the tubes reached a depth of 100 cm. Installation of the tubes at 45° from vertical has been shown to maximise root capture for some grass and tree species (Johnson *et al.*, 2001); however, due to the aerial roots of *Rhizophora*, this was not possible at our sites. Our measurements of fine root production may be conservative as a consequence; however, our installation procedure was consistent across our three plots. In all three sites, we allowed the soil to settle around the tubes for more than 5 months (March to September 2018) between initial installation and first data collection. Doing so helped limit fine root production artefacts, such as abnormal fine root production occurring in response to the severing of roots or in response to nutrients released during soil tube installation (Johnson *et al.*, 2001; Iversen *et al.*, 2012).

We took monthly measurements from September to December 2018. Our measurement period encompasses the end of the monsoon season and the beginning of the dry season. All

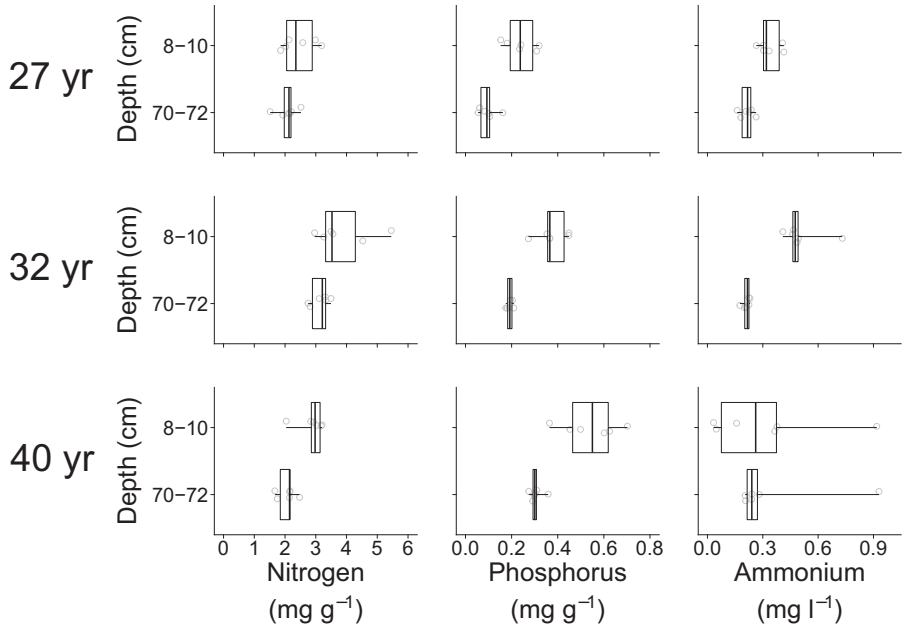

Fig. 2 Soil total nitrogen, total phosphorus and ammonium concentrations across the study sites. Heavy bars indicate sample medians; box ends indicate upper and lower quartiles; whiskers extend to maxima and minima of each sample. Open grey circles indicate individual measurements, with vertical jitter to reduce overwriting.

measurements were carried out at low tide and in the morning. In total, we measured root growth during three periods: September–October, October–November and November–December. The root production measurements were made over 3 d consecutively each month. The 32-yr-old site was measured during the first day, the 40-yr-old site during the second day and the 27-yr-old site during the third day. The time between measurements were therefore similar between sites. We considered root growth to mean either the longitudinal extension of existing roots, or the appearance of new roots, between the measurement periods (Fig. 3) (Johnson *et al.*, 2001; Iversen *et al.*, 2012). Due to the large number of roots, we limited our measurements of root growth to four depth intervals: 8–10, 30–32, 50–52 and 70–72 cm ($n = 4$). The depth interval of 2 cm is the length of the minirhizotron pictures (20 × 17 mm). We took two pictures at each depth interval at bearings of 0° (due north) and 180° (due south) for all minirhizotrons (from this point forwards referred as measurement direction, $n = 2$) on all sampling dates ($n = 4$). Lengths and diameters of individual roots were traced using a labour-intensive, manual procedure (see Arnaud *et al.*, 2019), which placed a constraint on the number of samples that could reasonably be processed.

For consistency, all minirhizotron images were collected and processed by the same operator (M. Arnaud) for all plots and on all dates, using a standardised protocol (Supporting Information Fig. S1) and Rootfly v.2.0.2 (Zeng *et al.*, 2007). We identified roots through their colour and shape (Fig. S1). We defined live roots as white or pale brown in colour (Fig. S1). In addition, some roots were covered with iron plaques, sediments or were partly black stained. Those roots were also included as live roots as long as they were in our depth of field or linked to white or pale brown roots (Fig. S1). Our root production quantification was conservative, because when roots did not meet those criteria, they were not included (Fig. S1). Measured changes in visible fine roots were converted into length increments to give mean daily fine root production in mm per square centimetre of soil observed through the minirhizotron window (mm cm^{-2} d^{-1}). We used this measure of production in our statistical analyses. Root length is the most commonly reported unit for rhizotron and minirhizotron studies, and has been shown to be more accurate than root numbers, despite being more time consuming to analyse (Johnson *et al.*, 2001). Changes in root area are less commonly reported than root length (Johnson *et al.*, 2001). We measured changes in root diameter, but these were very small or indistinct from zero. Indeed, the change of diameter of fine roots was difficult to measure accurately with Rootfly. More than 90% of all measured roots had a diameter < 1 mm. Although minirhizotrons sample roots up to 2–5 mm in diameter, their small sampling area might bias the relative proportion of fine roots toward the most common roots, the finest roots (< 1 mm, Taylor *et al.*, 2013). We have not attempted to convert our data into mass of production per unit volume of soil because further methodological developments are required for such a conversion. In particular, there is a need to define the depth of field of the observation (Iversen *et al.*, 2012), and to establish a relationship between root length and biomass for different root types (Iversen *et al.*, 2012; McCormack *et al.*, 2015). In total, the dataset we report here comprises 504 fine root production measurements, from 672 minirhizotron images of fine roots. The first sampling composed of 168 images was to estimate the initial standing root length.

Statistical analyses

To test our hypotheses (see the section 'Aim and hypotheses'), we developed a statistical model to describe our fine root production

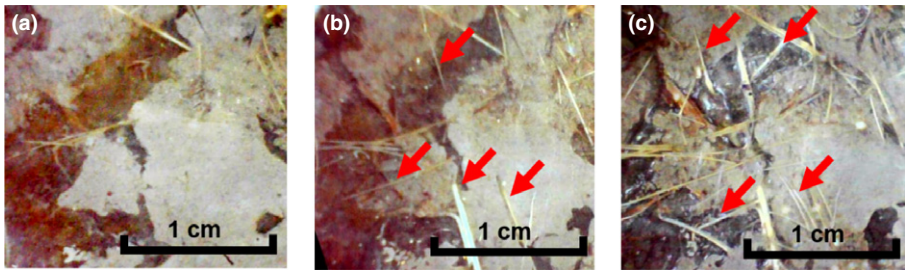

Fig. 3 Repeat images from the EnRoot minirhizotron showing temporal development of a belowground scene. (a) initial image after the settling period, >5 months since installation; (b) 1 month after initial image; and (c) 2 months after initial image. Red arrows indicate selected new roots that appeared since the previous image. Note that the minirhizotron images have been cropped around the area of interest.

measurements in terms of two categorical predictor variables, stand age (henceforth, *age*) and depth interval (*depth*), and their two-way interaction *age* × *depth* (Notes S1). Preliminary analyses showed our response variable, *productivity*, to be highly positively skewed, with a large proportion of zero measurements. Such a situation violates the assumptions of equal variance and normally distributed residuals required using standard ANOVA techniques. We used the glmmTMB function from the R package GLMMTMB (Brooks *et al.*, 2017) to fit a generalised linear mixed model (GLMM) to our data. To account for the zero-inflated response variable we specified a log link function, and we used a Tweedie distribution to represent the probability distribution of residuals.

Our measurements are grouped according to the minirhizotron tubes in which we took them, meaning that any within-site spatial patterns in fine root production introduce the potential for a hierarchical structure in our data. It is possible that our repeated measurements of production exhibited greater similarity within minirhizotron tubes than between them for any given combination of *age* and *depth*. We incorporated random effects into our GLMM to account for any such artefact. We assigned random intercepts, the subjects of which were the individual minirhizotron tube (*tube*). We also included random intercepts, with the measurement period (*month*) as the subject, to account for any temporal changes between our repeated measures. The depth interval and measurement direction were also nested within each tube across sites. We experimented with specifying other random intercepts that vary with depth and measurement direction, but these resulted in models that were numerically intractable and failed to converge so we omitted them from the final model. As a result, our sampling design may contain some artefacts of pseudoreplication that we have not been able to account for fully in our model specification. Marginal *P*-values and the exact values of estimated marginal means should therefore be interpreted cautiously. Similarly, all model combinations with random slopes were numerically intractable and failed to converge. The final model includes random intercepts according to *tube* and *month*. The R code to fit our final model is available in the Supporting Information.

We analysed the GLMM using the ANOVA function from the R package CAR (Fox & Weisberg, 2019). We used a Type-III sum-of-squares to analyse the significance of the main effects and their interaction based on chi-squared tests of their fitted values. We used the *emmeans* function from the R package EMMEANS (Lenth, 2020) to conduct Tukey post hoc comparisons between estimated marginal means for all pairs of samples.

Results

Measured mean fine root production decreased monotonically with increases in both age and depth. Averaged across all depths, marginal means of measured fine root production declined from 0.124 mm cm^{-2} d^{-1} (mean daily root length increment per square centimetre of soil observed through the minirhizotron windows) in the 27-yr-old site, to 0.063 mm cm^{-2} d^{-1} in the 32-yr-old site, and 0.017 mm cm^{-2} d^{-1} in the 40-yr-old site. Averaged across all stand ages, marginal means of measured fine root production declined from 0.172 mm cm^{-2} d^{-1} in the 8–10 cm depth interval, to 0.074 mm cm^{-2} d^{-1} at 30–32 cm depth, 0.013 mm cm^{-2} d^{-1} at 50–52 cm, and 0.013 mm cm^{-2} d^{-1} at 70–72 cm (Table 2). Fine root production in deep soil layers was not negligible compared with the 8–10 cm soil layer. For the 27-yr-old site, root production in the 30–32 and 50–52 cm layers represented 38.6% and 2.5% of that in the 8–10 cm soil layer, respectively. All fine root production measurements for the 70–72 soil layers were equal to zero for the 27-yr-old site. For the 32-yr-old site, root production in the 30–32, 50–52 and 70–72 cm layers represented 49.6%, 8.7% and 11% of that in the 8–10 cm soil layer, respectively. For the 40-yr-old site, root production in the 30–32, 50–52 and 70–72 cm layers was not significantly different ($P > 0.05$) from that in the 8–10 cm soil layer. Fine root production in the 30–32, 50–52 and 70–72 cm layers represented 89.3%, 129.3% and 143.3% of that in the 8–10 cm soil layer, respectively. More than 90% of all measured roots had a diameter < 1 mm.

Analysis of our GLMM indicated that both main effects, *age* and *depth*, and their two-way interaction, *age* × *depth* are all highly significant (Table 2). The significant interaction term is influential upon the model's predictions, and the estimated marginal means for the 12 factorial combinations of *age* and *depth* are skilful predictors of the measured marginal means (Table S1). However, the strong interaction between *age* and *depth* means that estimated marginal means are not skilful predictors of measured marginal means for some levels of the two main factors (Table S1). Therefore, we do not consider estimated marginal means or post hoc effects for the main effects of *age* and *depth*, and instead focussed on the pairwise comparison of the 12

Table 2 Summary of ANOVA performed on fixed effects in our GLMM.

Variable	Chi-squared	df	Significance
Intercept	10.045	1	0.002
Age	25.516	2	< 0.001
Depth	82.501	3	< 0.001
Age × depth	57.817	6	< 0.001

Significance calculated from chi-squared statistics using Type-III sum-of-squares. See also Fig. 4. AICc = −40.941.

factorial combinations of these two variables represented by the interaction.

The interaction *age* × *depth* represents the fact that fine root production in the shallowest layers declines with increasing stand age (Fig. 4). In the 27-yr-old site, rapid production of fine roots near the surface declined strongly and significantly with depth. The shallowest depth interval at this site, 8–10 cm, exhibited the greatest measured (0.352 mm cm^{-2} d^{-1}) and estimated (0.251 mm cm^{-2} d^{-1}) mean fine root production values in the entire study. At 30–32 cm depth in the same site, measured and estimated mean production declined to 0.136 and 0.101 mm cm^{-2} d^{-1}, respectively. By the 50–52 cm depth in this site, measured mean fine root production had fallen to 0.009 mm cm^{-2} d^{-1}, the lowest of any nonzero sample in the entire study; while estimated mean fine root production was 0.007 mm cm^{-2} d^{-1}, the second-lowest estimated mean. Production differs significantly between all of the three shallowest depth intervals (Fig. 4). The depth interval 70–72 cm at this youngest site was the only sample in the study for which we observed no fine root production whatsoever during the entire study period.

By contrast, the oldest site, 40 yr old during our field sampling, exhibited no significant depth variation in fine root production (Fig. 4). Fine root production rates are low at all depths, with measured marginal means between 0.013 and 0.022 mm cm^{-2} d^{-1}; and estimated marginal means between 0.005 and 0.014 mm cm^{-2} d^{-1}. The 32-yr-old site exhibited an intermediate behaviour between that of the youngest and oldest sites, with a significant decline in production with depth between some, but not all, pairs of depth intervals. Generally, the greatest contrasts in production occur when comparing shallow depth intervals in the 27-yr-old and 32-yr-old sites to deeper layers in those sites, or to any depths in the 40-yr-old site (Fig 4).

The inclusion of a random intercept for tube led to a highly significant improvement in model performance compared with a null model without this effect, measured by treating the difference in Akaike's Corrected Information Criterion (AIC_C) between the models as a chi-squared statistic (change in AICc = −46.489, df = 1, $P \approx 10^{-12}$). This result indicated important differences between individual minirhizotron tubes, an expression of spatial heterogeneity in fine root production. By contrast, the addition of a random intercept for measurement period, *month*, to a null model was highly nonsignificant (change in AICc = +1.700, df = 1, $P > 0.2$), indicating no appreciable effect of seasonality upon fine root production during our study period, nor any artefacts from postinstallation settling.

Discussion

The overall rate and depth distribution of fine root production varied with reforested mangrove stand age. These results have direct implications for: (1) predicting fine root production and SOM dynamics in mangroves following reforestation; and (2) understanding the vulnerability of reforested mangroves to future sea-level change, through their ability to occupy vertical accommodation space by accumulating fine roots.

Time since reforestation

The apparent age-related decrease of fine root production in shallow layers is in accordance with the developmental trajectory of natural mangroves, in which tree density declines gradually after stand maturity until senescence at *c.* 70 yr (Jimenez *et al.*, 1985; Fromard *et al.*, 1998; Alongi, 2009; Walcker *et al.*, 2018). Earlier work in natural mangrove forests showed that, as mangrove stands mature, tree density declines, a phenomenon known as self-thinning (Fromard *et al.*, 1998; Salmo *et al.*, 2013). We measured a similar decline of tree density with age (Table 1), and we visually observed (but did not measure) an increase of the size of individual trees with increasing stand age. Average tree densities at our sites, between 0.09 and 0.37 individuals m^{-2}, are in the range of those reported for undisturbed *Rhizophora* sp. stands (Sasmito *et al.*, 2020).

An association between tree density and fine root production has previously been reported in natural mangroves (Adame *et al.*, 2014) and in other (nonmangrove) secondary forests (Idol *et al.*, 2000; Law *et al.*, 2003). Our results are consistent with this relationship, showing a decline of fine root production in older mangroves with lower tree density. Canopy closure represents the starting point for the decline of fine root production in nonmangrove secondary forests (Idol *et al.*, 2000; Law *et al.*, 2003), and it might represent a similar starting point in mangroves, although more evidence is needed to test this hypothesis. Perez-Ceballos *et al.* (2018) observed an increase in mangrove fine root production before canopy closure, in a similar manner to nonmangrove secondary forests, with lower production in 2-yr-old replanted mangroves than in 3-yr-old replanted mangroves. The mangroves studied by Perez-Ceballos *et al.* (2018) were likely to be in an early pioneer stage, during which aboveground production is also known to increase; whereas our sites, which are substantially older, have reached maturity and begun to self-thin.

Some previous work reporting self-thinning has revealed an increase of aboveground biomass with age caused by an increase in the mass of individual trees (Analuddin *et al.*, 2009; Deshar *et al.*, 2012; Walcker *et al.*, 2018). Therefore, it is unclear whether older (larger) trees produce fewer fine roots per unit of aboveground biomass compared with younger trees; whether fine roots live longer in older stands; or whether the heterogeneity of root distribution changes with tree age. All of these hypotheses remain to be tested.

Tamooh *et al.* (2008) studied root biomass in individual trees across two mangrove chronosequences in Kenya. They found that the biomass of fine roots, which they defined as those with

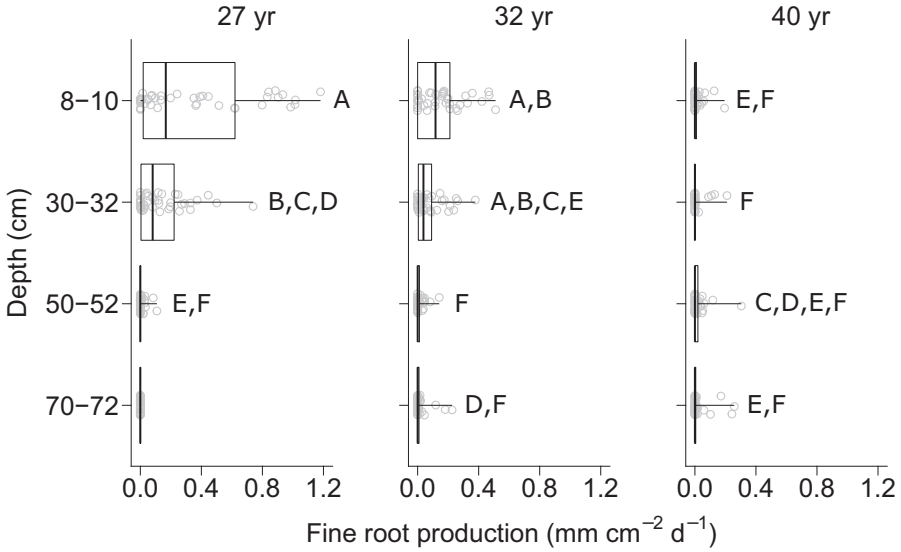

Fig. 4 Boxplots showing measured fine root (< 2 mm) production according to factorial combinations of stand age and depth increment. Fine root production is reported as mean daily root length increment per square centimetre of soil observed through the minirhizotron window (mm cm^{-2} d^{-1}). Heavy bars indicate sample medians; box ends indicate upper and lower quartiles; whiskers extend to maxima and minima of each sample. Open grey circles indicate individual measurements, with vertical jitter to reduce overwriting. Compact letter display indicates significant groupings amongst samples according to Tukey post hoc tests ($P < 0.05$ threshold).

diameters of < 5 mm, increased with age in *Rhizophora* spp. plots that were 6 and 12 yr old, and did not change with age in *Sonneratia* spp. plots that were 9 and 12 yr old. However, studies of root production and root biomass have often shown poor relationships between the two (Castañeda-Moya *et al.*, 2011; Adame *et al.*, 2014; Perez-Ceballos *et al.*, 2018). The age at which the decline of fine root production begins may depend on other factors such as inundation frequency and nutrient availability (Naidoo, 2009; Castañeda-Moya *et al.*, 2011; Adame *et al.*, 2014; Cormier *et al.*, 2015; Poungparn *et al.*, 2016; Perez-Ceballos *et al.*, 2018; Sánchez *et al.*, 2005; Torres *et al.*, 2019).

Soil nitrogen, ammonium and phosphorus have been shown to decline with mangrove stand age (Alongi *et al.*, 2004; Lovelock & Reef, 2020), and these growth-limiting nutrients are well known to control fine root production in mangroves (Feller *et al.*, 2003; Naidoo, 2009; Castañeda-Moya *et al.*, 2011; Cormier *et al.*, 2015) and other ecosystems (Pregitzer *et al.*, 1993; Yuan & Chen, 2010). We were unable to test the influence of nutrients on fine root production as part of our formal statistical modelling because our destructive sampling for soil nutrient measurements in the field meant that those data are not associated directly with specific root growth measurements. Nonetheless, neither total soil nitrogen nor ammonium decreased monotonically with stand age along our chronosequence, and therefore appear unlikely to be responsible for the between-site differences in fine root production. Similarly, Alongi *et al.* (1998) observed no difference in soil ammonium content between 15-yr-old and 60-yr-old reforested *Rhizophora* mangroves. Although total phosphorus differed significantly between our sites, it increased with stand age, and therefore also cannot be used to explain the age-related decrease in fine root production in shallow soil layers.

Depth variation

The depth-related decline in fine root production at the 27-yr-old and 32-yr-old sites is consistent with previous observations from natural mangroves (Castañeda-Moya *et al.*, 2011; Xiong *et al.*, 2017). In our sites, this vertical pattern of fine root production may result from higher concentrations of soil nutrients in the surface than deeper in the soil (McKee, 2001; Adame *et al.*, 2014).

Soil total nitrogen and phosphorus decreased significantly with depth at all three sites. Although this would appear consistent with the depth decline in fine root production in the 27-yr-old and 32-yr-old sites, it would seem unable to account for the lack of variation in the 40-yr-old site, where fine root production was low at all depths. By contrast, ammonium decreased with depth in the two youngest sites as did the fine root production, and exhibited no significant depth variation in the 40-yr-old site (it showed a nonsignificant increase with depth). The depth distributions of fine root production in all three sites are therefore consistent with depth distributions of soil ammonium, which has been shown to be the primary source of nitrogen in some mangroves (Reef *et al.*, 2010). However, the fact that nutrients could not be included in our GLMM due to the need for destructive soil sampling means that we must acknowledge the possibility of other factors in determining the depth distribution of fine root productivity. These include, but are not limited to, the roles of other nutrients, soil structure and aboveground biomass (Castañeda-Moya *et al.*, 2011; Xiong *et al.*, 2017; Muhammad-Nor *et al.*, 2019).

Reef & Lovelock (2015) proposed the possibility of an opportunistic distribution of fine roots at shallow depths to increase uptake of precipitation-derived fresh water. Although this theory would appear consistent with our observed root distributions at the 27- and 32-yr-old sites, the same pattern is not evident in our

40-yr-old site, so such a mechanism does not appear to offer a general explanation across our sites. Furthermore, our repeated measures of fine root production exhibited no significant seasonal change despite large temporal variations in rainfall during the study period (see Section 'Environmental conditions at the study sites'). Another potential explanation for the depth variation of root production in the youngest two sites could be a variation of redox-potential with soil depth (McKee, 1993; Gleason *et al.*, 2003). It is likely that the shallowest soil layers are more aerobic than deeper layers due to radial loss of oxygen by roots and oxygenation of the soil through animal burrows (e.g. crabs and mud lobsters).

Implications for mangrove carbon cycling

The decay of SOM and fine root production are thought to be the dominant controls on the accumulation of SOM following mangrove reforestation (e.g. Alongi *et al.*, 1998). The accumulation rate of autochthonous soil organic carbon has been shown to decrease after mangrove stands reach maturity (Marchand, 2017), mirroring the age-related decline of near-surface fine root production observed in our sites, and adding to the evidence that fine root production controls SOM accumulation. Similarly, in a meta-analysis, Lunstrum & Chen (2014) showed that the rate of belowground carbon accumulation decreased after mangroves reached 5 yr old. Additionally, fine root production is associated with exudation from live roots, which can influence SOM decay. Labile carbon from exudates can promote decomposition by priming old carbon (Kuzyakov *et al.*, 2000). Age-related changes to depth distributions of fine roots and their associated exudates seem likely to have consequences for carbon residence time in mangrove soils. Predictions of future changes in mangrove carbon stores and sinks (e.g. Lovelock & Reef, 2020) may, therefore, be materially improved by accounting for the effects of mangrove age upon the overall rate and depth distribution of fine root production.

Although mangrove carbon budgets sometimes include measurements of fine root production as deep as 90 cm, it is common to consider only the uppermost 30 cm of the soil profile (Bouillon *et al.*, 2008; Twilley *et al.*, 2017). However, we found that fine root production in deeper layers (i.e. 30–32, 50–52 and 70–72 cm depths) can be a nontrivial fraction of that in the shallowest soil layer, and is likely to represent an important contribution to total fine root production. Our sampling covered only a small fraction of each of our study sites and the distribution of root production with depth was variable within sites. Yet, the appreciable fraction of root production that we found deeper than 30 cm depth is in accordance with other studies investigating depth distributions of mangrove root production (Castañeda-Moya *et al.*, 2011; Muhammad-Nor *et al.*, 2019). The only two studies reporting root production at depths below 30 cm found a significant amount of fine root production in those soil layers (up to 45% found by Castañeda-Moya *et al.*, 2011; and up to 43% found by Muhammad-Nor *et al.*, 2019; both studies used the ingrowth core method). Additionally, live roots have been commonly reported below 30 cm in mangrove soils (Komiyama *et al.*, 1987, 2000; Tamooh *et al.*, 2008; Adame *et al.*, 2017). Our observations of variations in fine root production are likely to be applicable to other sites, as *Rhizophora* is distributed globally in mangroves, and holds the second largest global stock of mangrove carbon (Atwood *et al.*, 2017). Therefore, the role of fine roots in the assessment of mangrove carbon budgets (Bouillon *et al.*, 2008; Alongi, 2009; Twilley *et al.*, 2017) may to date have been grossly underestimated.

Conclusions

(**1**) Production of fine roots in shallow soil layers decreased with the age of reforested mangrove stands. While similar patterns have previously been observed in aboveground production, our findings are, to our knowledge, the first to demonstrate it for belowground production.
(**2**) Fine root production decreased with depth in the two youngest sites, likely due to nutrient limitations; but this depth variation was not apparent in the oldest site, where fine root production was low for all depths. The vertical pattern of fine root production may have important implications for understanding mangrove soil accumulation and rhizosphere processes.
(**3**) Large amounts of fine root production were found deeper than 30 cm in the soil, yet these layers are commonly omitted from mangrove carbon budget calculations. Our results highlight the need to give due consideration to these deeper layers.

Acknowledgements

The processing and the analysis of the data, as well as writing the manuscript were self-funded by M. Arnaud. This research received funding from National Geographic (CP134ER-17), the Royal Geographical Society with the Institute of British Geographers, the Estuarine & Coastal Sciences Association (Charles Boyden Fund), and the University of Leeds (International Mobility Grant, Climate Bursary Grant and Leeds University PhD Scholarship), all awarded to M. Arnaud. We thank David Ashley from the School of Geography at the University of Leeds for his help with the laboratory analysis and instrumentation support. We also thank the students and staff of the Vietnamese National University of Ho Chí Minh City for their support in the field. The management committee and staff of the Cần Giờ Park gave permission for access to, and use of, the field site and are thanked for their support during the field work, especially Mr Nghia, Mr Sinh, Mr Hung, Mr Hoan, Mr Sang and Mr Kiet.

Author contributions

MA was responsible for the study design, data collection and analysis, as well as writing the manuscript. PJM also contributed substantially to the data analysis. PJM, AJB, HD and TTN contributed to the study design, data collection and to manuscript development.

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Data availability

Dataset https://doi.org/10.7910/DVN/PFUWRT.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Fine root identification protocol.

Notes S1 R script describing the GLMM model.

Table S1 Comparison of measured and estimated marginal means of fine root production.

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