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Introduction of *Dalbergia odorifera* enhances nitrogen absorption on *Eucalyptus* through stimulating microbially mediated soil nitrogen-cycling

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Abstract

Background: There is substantial evidence that *Eucalyptus* for nitrogen (N) absorption and increasing the growth benefit from the introduction of N-fixing species, but the underlying mechanisms for microbially mediated soil N cycling remains unclear.

Methods: We investigated the changes of soil pH, soil water content (SWC), soil organic carbon (SOC), total N (TN), inorganic N ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$), microbial biomass and three N-degrading enzyme activities as well as the biomass and N productivity of *Eucalyptus* between a pure *Eucalyptus urophylla* × *grandis* plantation (PP) and a mixed *Dalbergia odorifera* and *Eucalyptus* plantation (MP) in Guangxi Zhuang Autonomous Region, China.

Results: Compared with the PP site, soil pH, SWC, SOC and TN in both seasons were significantly higher at the MP site, which in turn enhanced microbial biomass and the activities of soil N-degrading enzymes. The stimulated microbial activity at the MP site likely accelerate soil N mineralization, providing more available N ($\text{NH}_4^+\text{-N}$ in both seasons and $\text{NO}_3^-\text{-N}$ in the wet-hot season) for *Eucalyptus* absorption. Overall, the N productivity of *Eucalyptus* at the MP site was increased by 19.7% and 21.9%, promoting the biomass increases of 15.1% and 19.2% in the dry-cold season and wet-hot season, respectively.

Conclusion: Our results reveal the importance of microbially mediated soil N cycling in the N absorption on *Eucalyptus*. Introduction of *D. odorifera* enhances *Eucalyptus* biomass and N productivity, improve soil N availability and increased soil C and N concentration, which hence can be considered to be an effective sustainable management option of *Eucalyptus* plantations.

Keywords: *Eucalyptus* plantations, Soil physical-chemical properties, Microbial biomass, Soil enzyme activities, Nitrogen availability

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Background

Eucalyptus is one of the most extensively planted commercial plantation timber genera in the world (Lino et al. 2016), and it has been introduced and cultivated in China since the 1970s. The area of *Eucalyptus* plantations in China has reached 4.6 million ha by 2014, of which almost half in Guangxi Zhuang Autonomous Region, China (Zhao et al. 2018). Currently, most *Eucalyptus* plantations are grown as monocultures and have been intensively managed in short-rotation with continuous cropping (Huang et al. 2017). However, due to high nitrogen (N) consumption with successive rotations, the monoculture plantations of *Eucalyptus* have rapidly depleted soil nutrients (especially N) and water (Liu et al. 1998; Sicardi et al. 2004). The massive application of N fertilizer is common to ensure high and sustainable stand production (Laclau et al. 2005), but this not only increases economic costs but also leads to soil acidification and contamination of groundwater, and other negative impacts on the environment (Goncalves et al. 1997). Consequently, seeking an optimal silvicultural practice to achieve N sustainable management of *Eucalyptus* plantations is in urgent need.

The N-fixing trees have been widely considered as being important in balancing N losses due to timber harvesting and in reducing the demand for fertilizer application in *Eucalyptus* plantations (May and Attiwill 2003). The growth of N-fixing plants relies largely on the fixed atmospheric-N that accounts for 10%–90% of the N used by the N-fixing species (Nygren and Leblanc 2015). Therefore, introduction of N-fixing species into *Eucalyptus* plantations would decrease the N uptake of trees from soil, in turn increasing soil N retention (Bouillet et al. 2008; Koutika et al. 2019). In addition, the N transfer probably can also occur via mycorrhizal associations from acacia to eucalypt (Paula et al. 2015; Oliveira et al. 2021), which allows *Eucalyptus* to benefit directly from symbiotic N fixation (Paula et al. 2015; Yao et al. 2019, 2021). In recent years, introducing N-fixing species to improve N absorption and biomass productivity on *Eucalyptus* has been a popular focus of research, but the experimental results remain contradictory depending on the selected N-fixing species and stand conditions (Forrester et al. 2006; Firn et al. 2007; Bouillet et al. 2013). Moreover, because of lack of understanding the underlying mechanisms of soil N cycling, it is still hard to predict how combinations of the N-fixing species and specific sites can lead to the best benefits, making the extension of this silvicultural practice remains difficult around the world (Forrester et al. 2006).

The advantages of *Eucalyptus* mixed with N_2 -fixing tree species are usually attributed to the increase in soil organic N source (Paula et al. 2015; Yao et al. 2019). However, it is important to note that most of these

increased organic N sources due to the introduction of N-fixing species becoming available to *Eucalyptus* have to be transformed to inorganic N by the microbial decomposition of the plant tissues and soil organic matter (Versini et al. 2016). Shift of microbial activity under the mixed plantation of *Eucalyptus* and N-fixing species would alter soil N cycling and availability, which ultimately improve the N absorption and growth on *Eucalyptus* (Huang et al. 2014). Microorganisms involve soil biogeochemical cycling mainly through producing specific extracellular enzymes, which has been confirmed by extensive researches (Huang et al. 2017). Soil N-degrading enzymes including leucine aminopeptidase (LAP), β -1,4-N-acetylglucosaminidase (NAG) and urease can serve as indicators of energy N demand (Schimel et al. 2017), which catalyze terminal reactions to depolymerize organic N (Sinsabaugh et al. 2008). Therefore, detecting the change of microbial biomass and soil N-degrading enzyme activities will help us to better understand the underlying mechanisms for microbially mediated soil N cycling under the mixed plantations of *Eucalyptus* and N-fixing species.

In recent years, a variety of N-fixing species such as *Acacia mangium* and *D. odorifera* were introduced to improve N absorption and productivity on *Eucalyptus* plantations in Guangxi (Yao et al. 2019). Previous work in pot experiment has observed a percentage of 6.6%–13.6% N transfer from *D. odorifera* to *Eucalyptus* and an increase of 20.4%–33.2% in the dry matter yields of *Eucalyptus* compared to the monoculture plantation (Yao et al. 2019). The aim of this study is to better understand the underlying mechanisms for microbially mediated soil N cycling when introducing *D. odorifera* into the *Eucalyptus* plantations. Specifically, we hypothesized that the introduction of *D. odorifera* would increase soil organic matter (SOC and TN) and improve soil pH and SWC, which in turn stimulated microbial activities (biomass and N-degrading enzyme activities) and increased N availability (NH_4^+ -N and NO_3^- -N), ultimately enhancing *Eucalyptus* N absorption and productivity. We also expected that the role and the driving factors of microbially mediated soil N cycling when introducing *D. odorifera* into the *Eucalyptus* plantations differ between in the dry-cold and wet-hot seasons.

Materials and methods

Experimental design

The study site was located at the Experimental Center of Tropical Forestry, Chinese Academy of Forestry (22°07' N, 106°93' E), Pingxiang City, Guangxi Zhuang Autonomous Region, China. The mean annual precipitation is around 1400 mm, falling mainly from April through September (Wang et al. 2010) and mean annual temperature is 21 °C. The soils were formed from granite, classified

as red soil in Chinese soil classification, equivalent to oxisol in USDA Soil Taxonomy (Huang et al. 2014, 2017). Prior to the current study, this area was covered by a planted *Acacia mangium* plantations, which was established in 2001 on a historically deforested hill and clear harvested in 2014.

In March 2015, a complete randomized block design with three replicates was established to compare a pure *E. urophylla* × *grandis* plantation (PP) and a mixed species plantation of *D. odorifera* and *E. urophylla* × *grandis* (MP) in a field experiment. The two species were planted alternately at 2-m spacing in the row, with 2.5 m between rows, giving a total stocking density of 2000 trees·ha⁻¹. These densities reflect the current densities in commercial plantations in South China. Each plot consisted in 15 m × 15 m and a 200-m buffer separates the PP and MP plantations, and were either pure plots of *E. urophylla* × *grandis* (100E) or mixed plots in a proportion of 1:1 of both species (50D:50E, the two species being alternately planted in the row, and between adjacent rows). The fertilizers applied on planting were 140 g N·plant⁻¹ (urea: CO (¹⁴NH₂)₂, buried at 20 cm from the each plant), as well as 18 g·plant⁻¹ K (KCl), 56 g P·plant⁻¹ (CaH₂PO₄) in both PP and MP plantations.

Sample of plants collected and N analysis

Five plants in each plot were harvested on March 20 and August 25, 2019, which including below-ground (i.e., roots) and above-ground. The trees of above-ground were separated into components: leaves, branches (living branches and dead branches), stem wood and stem bark. The stem of each tree was sawn into 2-m sections according to Monsie's stratified clip method (You et al. 2018). However, much of the root growth was shallow and lateral, it was difficult to distinguish the fine roots of one tree from those of another. To address this problem, a large soil pit of 1.5-m diameter and 1.0-m depth was excavated around each target tree. All the materials were collected and weighed up fresh weight immediately, and then 500 g of every composition sample was taken in laboratory.

The harvested material was dried at 65 °C until constant dry weight period for the biomass analyses. The dried plant material was ground in a ball mill (< 0.1 mm) for the N concentration analyses with a continuous-flow chemical analyzer (AA3) after the degradation using 10 mL H₂SO₄. The biomass and N accumulation of *E. urophylla* × *grandis* in the pot was the sum of leaves, stem, branches, bark and roots.

Sample of soils collected and analysis

On March 19 and August 24, representing the dry-cool season and the wet-hot seasons of the year 2019, respectively, five soil samples were randomly collected

from each plot at 0–20 cm depth to investigate the changes of soil pH, soil water content (SWC), soil organic carbon (SOC), total N (TN), inorganic N (ammonium nitrogen: NH₄⁺-N and nitrate nitrogen: NO₃⁻-N), microbial biomass and three N-degrading enzyme activities. During sampling within a plot, the corer was wiped clean of obvious soil particles with paper towel. All samples were stored at -20 °C and sieved < 2 mm to remove visible stones, soil animals, roots and plant materials prior to analysis pH, ammonium (NH₄⁺-N) and nitrate nitrogen (NO₃⁻-N). Some of the air dried and ball milled sample (sieved < 0.2 mm) were used for the concentration of total nitrogen TN, soil microbial biomass carbon (MBC) and nitrogen (MBN) and (SOC) analyses.

The pH of the soil samples was measured in a 1:2.5 soil/water suspension. SOC was determined by dichromate oxidation and titration with ferrous ammonium sulfate, the TN concentration was determined by a continuous flow chemical analyzer (AA3) and followed by detection of NH₄⁺-N and NO₃⁻-N. Data was also collected on C/N ratio of soil (C/Nsoil).

MBC and MBN were measured by fumigation-extraction, using 0.5 M K₂SO₄ as the extraction agent (Vance et al. 1987), with a total organic carbon analyzer (1020A; OI, College Station, TX, USA), and were calculated by Vance et al. (1987).

In addition, soil microbial function was expressed by the soil extracellular enzyme activities, involved NAG, LAP and Urease, respectively. We determined the soil enzyme activities using the conventional p-nitrophenol (pNP) assays (Baldrian 2009). The enzyme activities were measured by 3 replicates for each soil sample and expressed as μmol per gram dry soil and incubation time.

Statistical analyses

Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Analysis of Variance (ANOVA) was used to determine the statistical significance ($\alpha = 0.05$) of stand type, season and their interactive effect on N accumulation and biomass of *Eucalyptus* as well as soil physico-chemical properties and microbial activity. Tukey's multiple comparison test (HSD) was conducted if significant effects of stand type or season was found. Pairwise relationships between plant N accumulation, soil physico-chemical properties and microbial activities were tested using Pearson correlation coefficients.

Structural equation modeling (SEM) is an advanced multivariate statistical technique that allows for hypotheses testing of complex path-relation networks (Grace et al. 2007). A structural equation modelling (SEM) approach was also used to test a conceptual model for

microbially mediated soil N cycling in the dry-cold and the wet-hot seasons. The SEM analysis was performed with the IBM SPSS Amos 20.0 using the maximum likelihood estimation method. Several tests were used to assess model fit: the Chi-square (χ^2)-test, comparative fit index (CFI) and root square mean error of approximation (RMSEM) (Arbuckle 2006).

Results

The biomass and N productivity of *E. urophylla* × *grandis*

The total biomass of *Eucalyptus* was significantly affected by stand type (Table S1), with greater at the MP than the PP sites in both seasons (Fig. 1a and b; $p < 0.05$). For the different organs of *Eucalyptus*, the biomass in branch and stem were significantly greater at the MP than PP sites in the wet-hot season, while in the dry-cold season only the leaf biomass were significantly greater at the MP than PP sites. At both PP and MP sites, the biomass of *Eucalyptus* in stems was greatest, and then followed by root, branch, bark and leaf in both seasons (Fig. 1a and b). The N productivity of the whole plant was significantly affected by season, stand type and their interaction (Table S1). Similar to biomass, the total N productivity of *Eucalyptus* was also significantly

greater at the MP than the PP sites in the both seasons (Fig. 1c and d). For the different organs of *Eucalyptus*, the N productivity in leaf, branch and root were significantly greater at the MP than PP sites in the wet-hot season, while in the dry-cold season only the leaf N productivity were significantly greater at the MP than PP sites. More importantly, the increased N productivity of *Eucalyptus* with the introduction of *D. odorifera* was relatively greater in leaf than in the other organs (Fig. 1c and d). The distribution of N productivity in different organs from high to low were stem, root, ranch, bark and leaf in the dry-cold season and were stem, branch, leaf, root and bark in the wet-hot season, respectively (Fig. 1c and d).

Soil physico-chemical properties

Soil pH had no significant difference between the dry-cold and wet-hot season, but was significantly higher at the MP site than at the PP site (Table 1). The concentrations of SOC, TN, NO_3^- -N and NH_4^+ -N as well as the ratio of SOC to TN (C/N) and SWC were significantly affected by stand type and season. SOC, TN, NH_4^+ -N and SWC were significantly higher at the MP than the PP sites and in the wet-hot season than dry-cold season

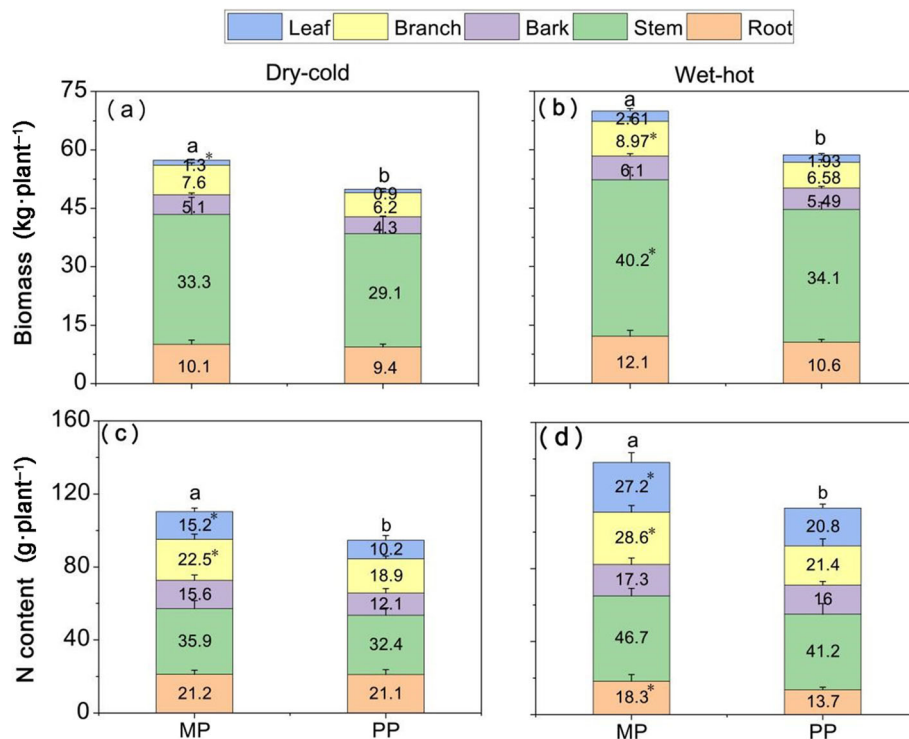


Fig. 1 The biomass and the N accumulation of *E. urophylla* × *grandis* on pure *Eucalyptus* and mixed with *N₂*-fixing species and *Eucalyptus*, respectively. **a, b** represent the biomass of *E. urophylla* × *grandis* and **c, d** represent the N content of *E. urophylla* × *grandis* in dry-cold season and wet-hot season, respectively. PP = pure *Eucalyptus* plantations; MP = mixed with *N₂*-fixing species *Eucalyptus* plantations. Different lowercase letters (**a** and **b**) on the bars are different significantly of the whole plant ($p < 0.05$) and * is different significantly in the same organ between the PP and MP site. Error bars indicate ± SE (n=4)

Table 1 Soil Physical chemical properties as affected by introduction N₂-fixing trees and seasons in *Eucalyptus* plantations. PP = pure *Eucalyptus* plantation; MP = mixed with N₂-fixing species and *Eucalyptus* plantation

Source of variation		pH	SWC (%)	SOC (g·kg ⁻¹)	TN (g·kg ⁻¹)	NO ₃ ⁻ -N (mg·kg ⁻¹)	NH ₄ ⁺ -N (mg·kg ⁻¹)	C/N
Dry-cold	MP	4.83 ± 0.12 a	18.63 ± 1.43 b	21.49 ± 3.25 b	1.34 ± 0.13 b	4.22 ± 0.46 b	16.99 ± 3.17 b	16.09 ± 2.03 b
	PP	4.56 ± 0.27 b	16.83 ± 0.86 c	18.96 ± 2.81 c	1.04 ± 0.15 c	4.09 ± 0.36 b	13.55 ± 2.01 c	18.78 ± 3.14 a
Wet-hot	MP	4.91 ± 0.16 a	21.48 ± 1.63 a	24.70 ± 3.62 a	1.56 ± 0.15 a	5.94 ± 0.88 a	17.46 ± 1.48 a	16.29 ± 3.69 b
	PP	4.60 ± 0.14 b	19.99 ± 1.11 b	20.85 ± 2.57 b	1.12 ± 0.10 c	4.25 ± 0.27 b	13.59 ± 1.56 c	18.15 ± 0.94 a
Season (S)		ns	64.832***	25.48***	100.852***	87.659***	144.709***	ns
Stand type (T)		7.844*	19.332***	18.631***	9.330**	48.121***	24.905***	7.844*
S × T		ns	ns	ns	8.522**	50.178***	13.667***	ns

ns, *, ** and *** = F-value not significant, significant at $p > 0.05$ and $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

($p < 0.05$ for all, Table 1). The soil NO₃⁻-N concentration at the MP site was higher than that at the PP site in the wet-hot season only (Table 1). Compared with the PP site, soil C/N ratio was significantly decreased at the MP site in both seasons (Table 1).

Microbial biomass and enzymes activities

Two-way ANOVAs showed that MBC and MBN were significantly affected by stand type and season ($p < 0.05$, Fig. 2), but no significant interactive effect was found ($p > 0.05$, Fig. 2). The MBC and MBN were significantly higher at the MP than the PP sites and in the wet-hot season than dry-cold season (Fig. 2a and b). However, the ratio of MBC/MBN did not significantly differ between the PP and MP sites in the both seasons (Fig. 2c). All of the soil N-degrading enzyme activities were also significantly affected by stand type and season ($p < 0.05$, Fig. 3). Specifically, only the activities of NAG and Urea in the wet-hot season were significantly higher ($p < 0.05$) at the MP than the PP sites (Fig. 3).

Relationships of soil microbial biomass and enzymes activities with soil and plant N pools

Soil pH did not correlated with any other properties in the dry-cold season, while in the wet-hot season it was positively correlated with SWC, SOC, TN, NAG activity and NO₃⁻-N (Table 2). SWC was positively correlated with MBC, the activities of NAG and Urea, and the N accumulation of *Eucalyptus* in the dry-cold season, and with SOC, TN, NAG activity, NH₄⁺-N, NO₃⁻-N and the N accumulation of *Eucalyptus* in the wet-hot season, respectively (Table 2). SOC also did not correlated with any other properties in the dry-cold season, but in the wet-hot season it was positively correlated with TN, MBC, all of N-degrading enzyme activities, NH₄⁺-N, NO₃⁻-N and the N accumulation of *Eucalyptus* in the wet-hot season. Soil TN content was positively correlated with MBN and the N accumulation of *Eucalyptus* in the dry-cool season, and with MBC, the activities of NAG and Urea, NO₃⁻-N and the N accumulation of *Eucalyptus* in the wet-hot season, respectively (Table 2).

MBC and MBN were positively correlated with all of the N-degrading enzyme activities in the wet-hot season except for the relationship between MBN and LAP activity (Table 2). MBC was also positively correlated with NH₄⁺-N, NO₃⁻-N and the N accumulation of *Eucalyptus* in the wet-hot season (Table 2). NAG activity was positively correlated with NH₄⁺-N, NO₃⁻-N and the N accumulation of *Eucalyptus* in both seasons (Table 2). LAP activity was positively correlated with NH₄⁺-N and the N accumulation of *Eucalyptus* in the wet-hot season but not in the dry-cold season, while Urea activity was positively correlated with NH₄⁺-N and the N accumulation of *Eucalyptus* in the dry-cold season but not in the wet-hot season (Table 2). Both NH₄⁺-N and NO₃⁻-N were positively correlated with the N accumulation of *Eucalyptus* in the wet-hot season, while in the dry-cold season only the NH₄⁺-N was positively correlated with the N accumulation of *Eucalyptus* (Table 2).

Pathway analysis for *Eucalyptus* to enhance N uptake

The structural equation model on the regulatory pathway of soil N dynamics well passed all the statistical tests on adequacy of microbial biomass and enzyme activities (Fig. 4a and b). Overall, soil pH, SWC, SOC and TN in both seasons were significantly higher at the MP than PP sites (Table 1), which together stimulated microbial biomass and most of soil N-related enzyme activities although the control mechanisms are different between seasons (Fig. 4a and b). The enhanced microbial activity likely accelerated soil N mineralization, providing more available N (NH₄⁺-N and NO₃⁻-N) for *Eucalyptus* absorption. Path analysis pointed to direct and positive controls of soil N availability (NH₄⁺-N and NO₃⁻-N) by the activity of soil N-related enzymes in both seasons, but the significant control by microbial biomass on the soil N availability was observed in the wet-hot season only. Soil pH and SWC positively correlated with microbial biomass in the dry-cold season only. SOC and TN positively corrected with both microbial biomass and enzyme activity in the dry-cold season and there was also a positive relationship between microbial biomass and

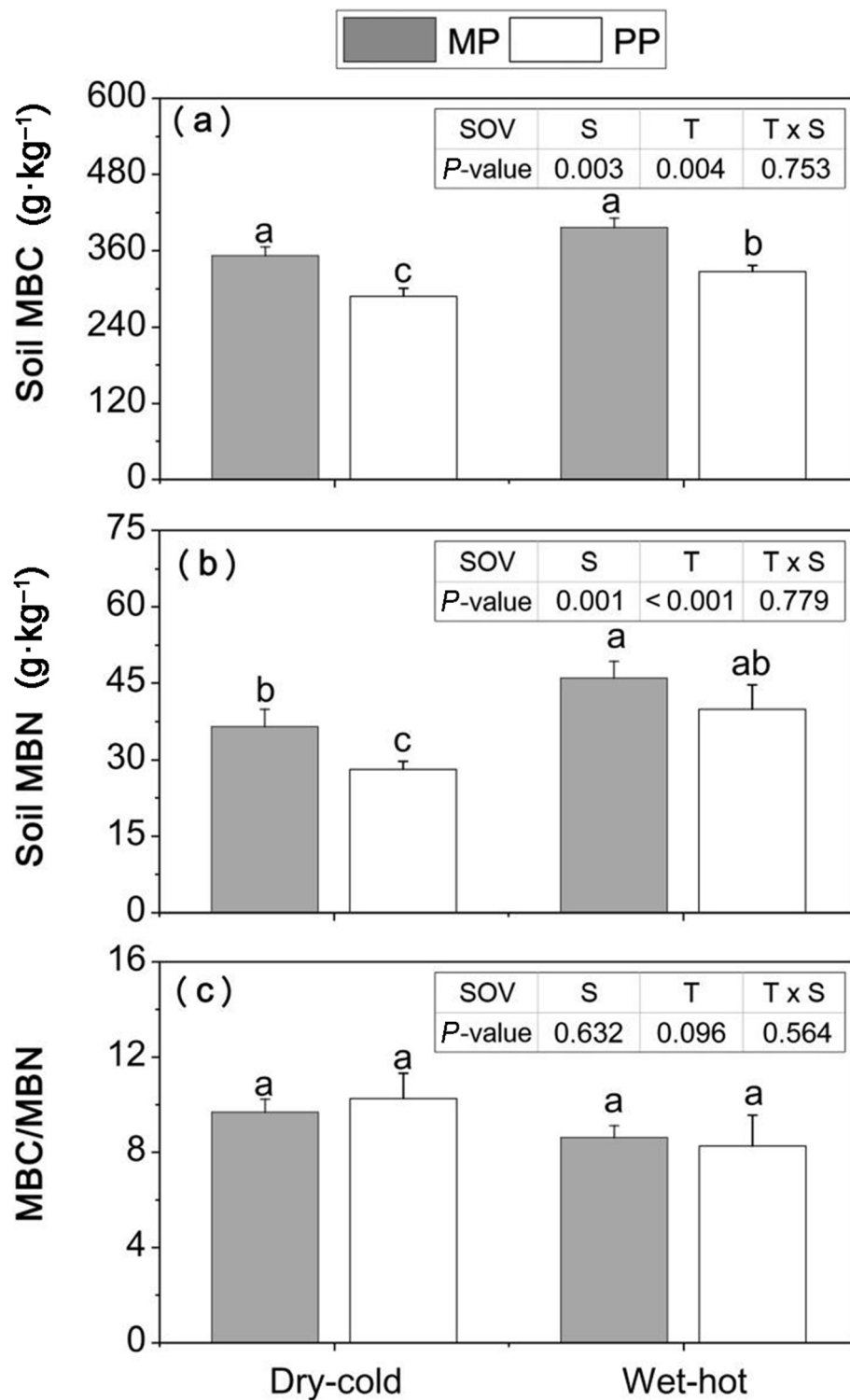


Fig. 2 The soil microbial biomass carbon (MBC) (a), microbial biomass nitrogen (MBN) (b) and MBC:MBN ratio (c) in the pure *Eucalyptus* plantations and mixed with *N₂-fixing species* and *Eucalyptus* plantations in both dry-cold and wet-hot seasons. PP = pure *Eucalyptus* plantations; MP = mixed with *N₂-fixing species* *Eucalyptus* plantations. Different lowercase letters (a, b and c) on the bars indicate significant differences under different treatments at $p < 0.05$. Error bars indicate \pm SE ($n = 3$). For the table in the top right-hand corner of figure, 'SOV', 'S', 'T' and 'S \times T' indicate that 'Source of variation', 'season', 'stand type' and 'interaction season \times stand type', P -values show that was not significant and significant affected for $p > 0.05$ and $p < 0.05$

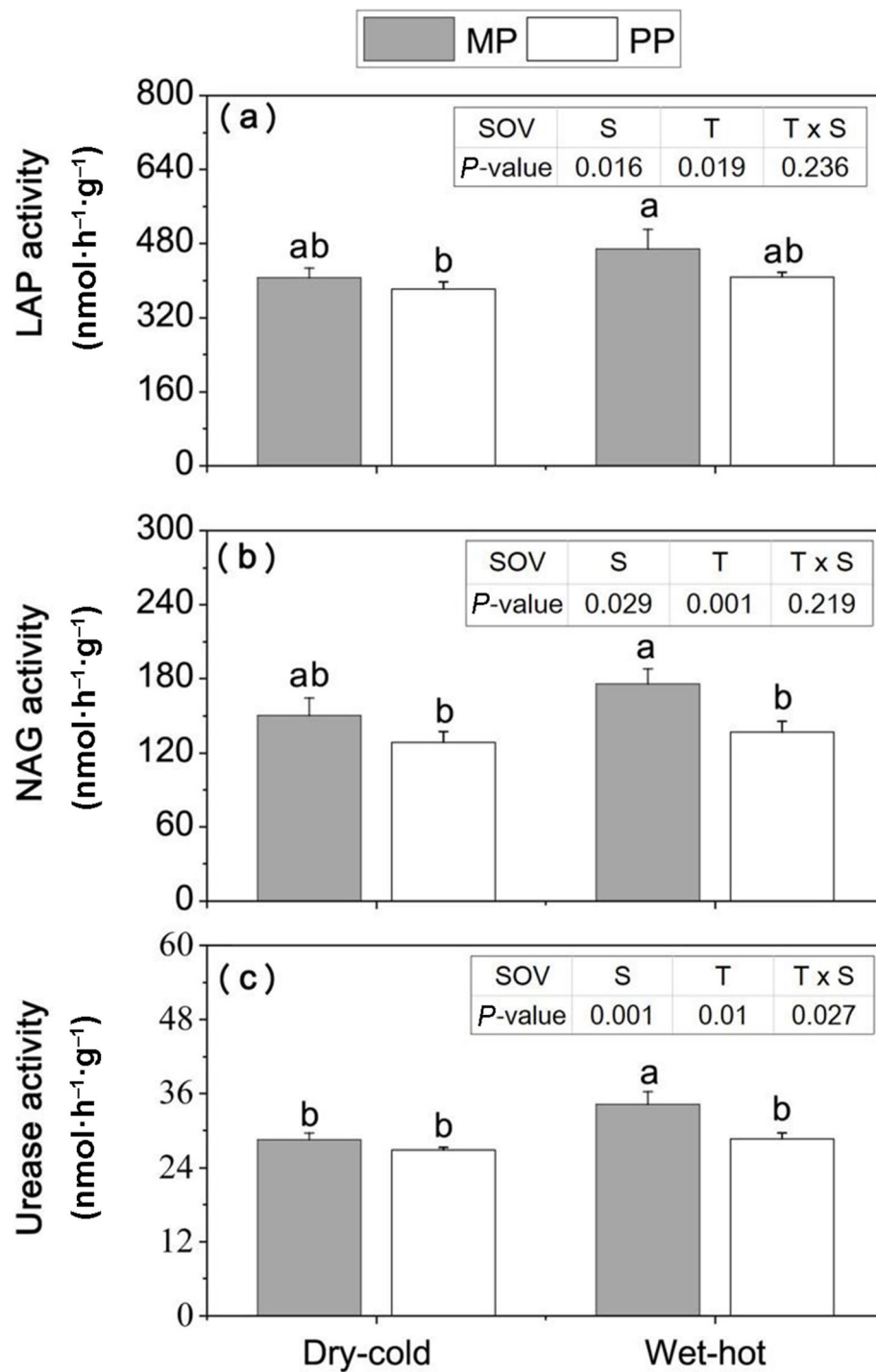


Fig. 3 The soil enzyme activities per gram of soil in the pure *Eucalyptus* plantations and mixed with *N₂-fixing species Eucalyptus* plantations in both dry-cold and wet-hot seasons. PP = pure *Eucalyptus* plantations; MP = mixed with *N₂-fixing species Eucalyptus* plantations. Different lowercase letters (a and b) on the bars indicate significant differences under different treatments at $p < 0.05$. Error bars indicate \pm SE ($n = 3$). For the table in the top right-hand corner of figure, 'SOV', 'S', 'T' and 'S \times T' indicate that 'Source of variation', 'season', 'stand type' and 'interaction season \times stand type', P -values show that was not significant and significant affected for $p > 0.05$ and $p < 0.05$

enzyme activity in both seasons. SOC and TN did not directly affect soil enzyme activity, but have an indirect effect via influencing microbial biomass.

Discussion

Mixed plantations of *Eucalyptus* and *N*-fixing species have been recognized as one of effective silvicultural

Table 2 The collection among the soil physical-chemical properties, soil enzymes, soil microbial biomass and N content in the plants

Source of variation	pH	SWC	SOC	TN	MBC	MBN	LAP	NAG	Urease	NH ₄ ⁺ -N	NO ₃ ⁻ -N	PN
pH		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SWC	0.976**		ns	ns	0.813*	ns	ns	0.895*	0.899*	ns	ns	0.946**
SOC	0.824*	0.857*		ns	ns	ns	ns	ns	ns	ns	ns	ns
TN	0.836*	0.87*	0.835*		ns	0.864*	ns	ns	ns	ns	ns	0.818*
MBC	ns	ns	0.888*	0.968**		ns	ns	ns	ns	ns	ns	ns
MBN	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns
LAP	ns	ns	0.866*	ns	0.812*	ns		ns	ns	ns	ns	ns
NAG	0.889*	0.947**	0.931**	0.908*	0.978**	0.825*	0.908**		0.994**	0.96**	0.858*	0.962**
Urease	ns	ns	0.850*	0.855*	0.866*	0.859*	ns	0.861**		0.935**	ns	0.967**
NH ₄ ⁺ -N	ns	0.859*	0.883*	ns	0.894*	ns	0.94**	0.946**	ns		0.897*	0.877*
NO ₃ ⁻ -N	0.902*	0.910*	0.947**	0.951**	0.952**	ns	ns	0.928*	ns	0.831*		ns
PN	ns	0.883*	0.873*	0.909**	0.976**	ns	0.894*	0.984**	ns	0.960**	0.893*	

SOC Soil organic carbon, TN Soil total nitrogen, NH₄⁺-N Ammonium nitrogen, NO₃⁻-N Nitrate nitrogen, PN Plant nitrogen content, MBC Microbial biomass carbon, MBN Microbial biomass nitrogen, LAP Leucine aminopeptidase, NAG β-1,4-N-acetylglucosaminidase. The values in the yellow area are in the wet-hot season and in the blue areas are in the dry-cool season. ns, * and ** = F-value not significant, significant at $p > 0.05$ and $p < 0.05$ and $p < 0.01$, respectively

practices to increase *Eucalyptus* productivity while maintaining soil fertility, compared to *Eucalyptus* monocultures (Epron et al. 2013; Tchichelle et al. 2017). For example, introducing *Acacia mangium* into *E. grandis* plantations provides better conditions for restoring the soil fertility and soil biodiversity, hence providing better sustainability of the cropping systems than pure *E. grandis* plantations (Garay et al. 2004). However, the results reported previously remain contradictory depending on the selected N-fixing species and stand conditions (Forrester et al. 2006; Firn et al. 2007; Bouillet et al. 2013). In this study, we discovered that the biomass and N productivity of *Eucalyptus* as well as soil TN and N availability were significantly enhanced by introducing *D. odorifera* into the *Eucalyptus* plantations. More importantly, the increased N accumulation of *Eucalyptus* with the introduction of *D. odorifera* was relatively greater in leaf than in the other organs (Fig. 1c and d). This may suggest that introduction of *D. odorifera* enhanced N uptake of *Eucalyptus*, and the N was primarily distributed to leaves so that better stimulate leaf photosynthesis and *Eucalyptus* growth.

The enhanced N productivity on *Eucalyptus* with introduction of *D. odorifera* should be attributed to the improved soil N availability, as N productivity of *Eucalyptus* was directly correlated with both soil NH₄⁺-N and NO₃⁻-N concentrations (Table 2). In agreement with our hypothesis, using structural equation model, we found that the higher soil organic matter (SOC and TN) and improved soil pH and SWC at the MP sites could indirectly enhance soil N availability via stimulating microbial activity (microbial biomass and enzyme activity). Increased soil TN under the mixed plantation of *Eucalyptus* and N₂-fixing species is common, as the growth of N-fixing plants relies largely on the fixed atmospheric-N (Paula et al. 2018). Therefore, introduction of N-fixing species into *Eucalyptus* plantations

would decrease the N uptake of trees from soil, in turn increasing soil N retention (Bouillet et al. 2008). Faster growth of N-fixing tree species and *Eucalyptus* due to higher N availability at the MP sites could also increase the input of plant residuals and hence enhance SOC and TN. Similar results were also found in other studies with a mixed plantation such as *E. globulus* and *A. mearnsii* in Australia (Forrester et al. 2005), *Eucalyptus grandis* and *Acacia mangium* in Brazil (Bini et al. 2013a, 2013b) and in Congo (Koutika et al. 2019, 2020), or *Eucalyptus regnans* and *Acacia dealbata* in southeastern Australia (Pfautsch et al. 2009). Soil organic matter has a huge specific surface area (mainly by humic substances) and a large number of negative charges (Gruba and Mulder 2015). Thus, increased SOC and TN at the MP sites may have a greater capacity in adsorbing cations, resulting in higher soil pH (Jiang et al. 2018). The increased SWC at the MP sites may be attributed to higher understory coverage of *D. odorifera* and the decreased water uptake by *Eucalyptus*. These findings suggest that the presence of leguminous trees in the system could improve stand conditions such as provide advantageous soil pH and SWC and more substrate availability (higher SOC and TN as well as decreased soil C:N) for microbial proliferation and the production of specific extracellular enzymes (Bowles et al. 2014). Therefore, soil microbial biomass and the activities of N-degrading enzymes in this study were generally higher at the MP than PP sites (Fig. 2). It is well known that increased microbial N-degrading enzyme activities reflect a higher rate of N mineralization and a higher level of N availability (Tabatabai et al. 2010). Although we have not determined the N mineral processes in this study, previous studies have frequently found that introducing N-fixing trees into *Eucalyptus* plantations can stimulate microbial activity (Pereira et al. 2018, 2019) to increase the rate of N mineralization (Voigtlaender et al. 2012, 2019).

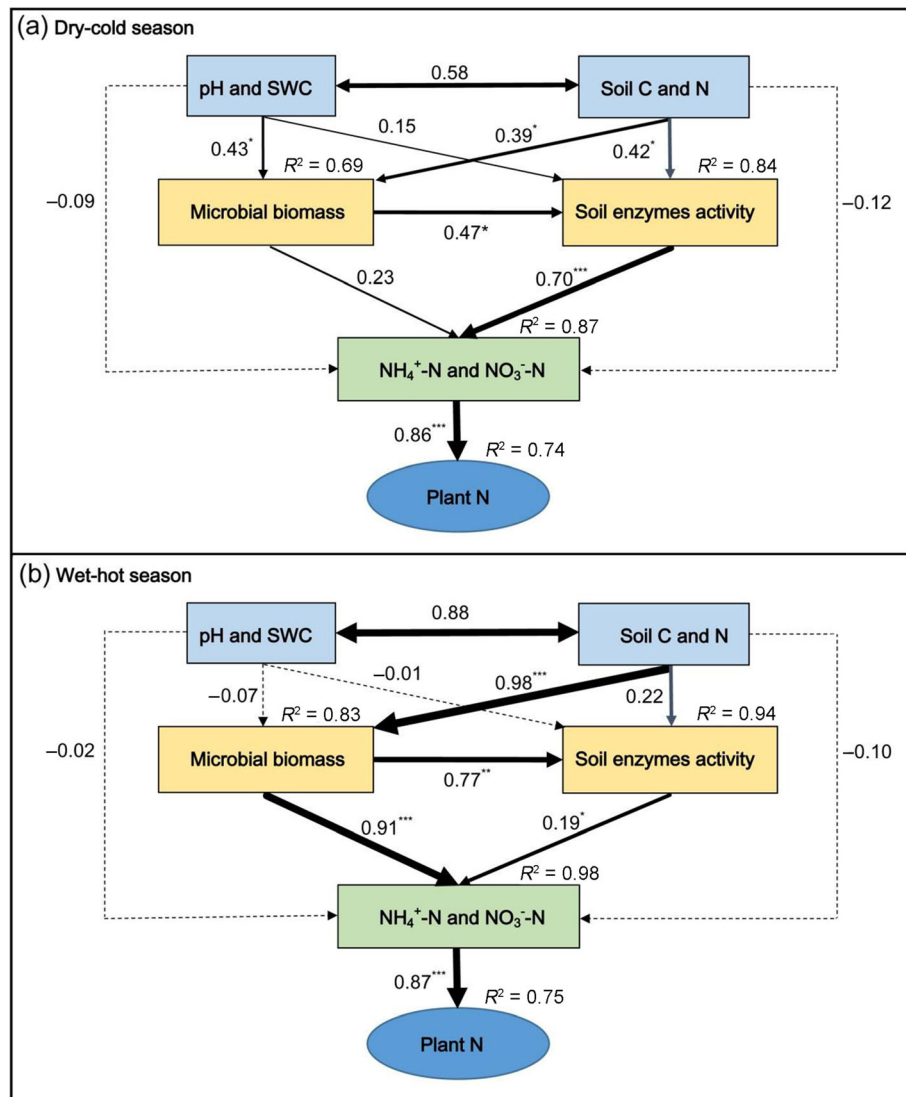


Fig. 4 Path model depicting the regulatory pathway of the controls of soil extracellular enzymes activities of soil physical-chemical properties (pH and soil water content: SWC), total nitrogen (TN), soil organic carbon (SOC) and microbial biomass (MBC and MBN) by the structural attributes to involve plant N absorption by plant. Where **(a)** represent structural equation model (SEM) in the dry-cold season for the model were $\chi^2 = 7.944$, $p = 0.056$, CMIN/df = 1.986, GFI = 0.900, RMSEA = 0.050, CFI = 0.933; and **(b)** represent SEM in the wet-hot season for the model were $\chi^2 = 4.663$, $p = 0.324$, CMIN/df = 1.166, GFI = 0.948, RMSEA = 0.026, CFI = 0.974. The black solid lines and dotted lines indicate significant positive and negative relationships, respectively; the thickness of the arrows reflect the degree of relationships, numbers at arrows are standardized path coefficients, and R^2 values indicate the variation of response variables explained by the model. *, ** and *** are different significantly at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

As expected, we also found that the driving factors and the role of microbially mediated soil N cycling when introducing *D. odorifera* into the *Eucalyptus* plantations differed between the seasons in this study. In the dry-cold season, soil microorganisms are usually subject to water limitation. Thus, increased SWC under the MP site could stimulate microbial biomass and the activity of N-degrading enzymes. This was supported by the positive relationship of SWC with MBC and the activities of NAG and Urease in the dry-cold season observed

in this study (Table 2). The structural equation model pointed out that increased soil organic matter also has positive effects on microbial biomass and the activity of N-degrading enzymes in the dry-cold season (Fig. 4a). However, we only found a positive relationship between TN and MBN in the dry-cold season (Table 2), suggesting that increased TN under the MP site might alleviate microbial N limitation and enhance microbial N assimilation. This in turn may explain why enhanced microbial biomass under the MP site did not directly contribute to

higher N availability in the dry-cold season (Fig. 4a). In the wet-hot season, both temperature and SWC are relatively higher at our study sites, thus increased SWC and pH under the MP site did not significantly influence microbial biomass and the activity of N-degrading enzymes (Table 2 and Fig. 4b). Alternatively, we found direct and positive effects of SOC and TN on microbial biomass but indirect effect on the activity of N-degrading enzymes (Table 2 and Fig. 4b), suggesting that microbial biomass and the activity of N-degrading enzymes in the wet-hot season was mainly limited by substrate availability in this study. Introduction of *D. odorifera* into *Eucalyptus* plantation significantly increased MBC but not MBN in the wet-hot season, thus having a direct and strong contribution to soil N availability (Fig. 4b). This was also supported by the positive relationships of MBC with $\text{NH}_4^+\text{-N}$ or $\text{NO}_3^-\text{-N}$ in the wet-hot season observed in this study (Table 2). Overall, introduction of *D. odorifera* into *Eucalyptus* plantation significantly increased soil N availability ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$) with higher in the wet-hot season than dry-cold season, despite the plant demand for N is greater in the wet-hot season. This suggests that the increased soil organic matter (SOC and TN) driven microbially mediated soil N cycling may play a relative stronger role in N accumulation and biomass productivity of *Eucalyptus* at the MP site.

Conclusion

Compared with the *Eucalyptus* monoculture, we found that the biomass and N productivity of *Eucalyptus* were significantly increased by introducing *D. odorifera* into the *Eucalyptus* plantation. Introduction of *D. odorifera* into the *Eucalyptus* plantation could increase soil pH, SWC, SOC and TN, which in turn enhanced microbial biomass and the activities of soil N-degrading enzymes as well as soil N availability. With the structural equation models, our results reveal the importance of microbially mediated soil N cycling in the biomass and N productivity of *Eucalyptus*, while highlight the seasonal difference in the driving factors. Soil microbial biomass and the activities of soil N-degrading enzymes in the dry-cold season were driven by increased SWC and TN, while in the wet-hot season they were mainly driven by increased SOC and TN. The mixed plantation also increased more soil N availability in the wet-hot season than dry-cold season. These findings can provide government and policy makers with useful tools to achieve N sustainable management in *Eucalyptus* plantations. However, further studies on the inclusion of litter quality and decomposition, soil next-generation sequencing as well as a longer time of evaluation are also essential to better understand the microbially mediated soil N cycling under the plantations of *Eucalyptus* and N-fixing species.

Abbreviations

TN: Total nitrogen; SOC: Soil organic carbon; SWC: Soil water content; PP: Pure *E. urophylla* × *grandis* plantation; MP: A mixed species plantation of *D. odorifera* and *E. urophylla* × *grandis*; MBC: Soil microbial biomass carbon; MBN: Soil microbial biomass nitrogen; LAP: Leucine aminopeptidase; NAG: β -1,4-N-acetylglucosaminidase

Supplementary Information

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Additional file 1: Table S1 The *P* values for season (S), stand type (T) and their interaction ANOVA effects on biomass and N content of *Eucalyptus urophylla* × *grandis*.

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Authors' contributions

All the authors designed the experiments; Yao X, Nong Z and Zhang Q carried out the experiments; Yao X, Ye S and Deng Q analyzed the experimental results, Yao X and Zhou H analyzed the data and developed analysis tools; Yao X, Ye S and Deng Q wrote the manuscript, and all authors read and approved the final manuscript.

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Not applicable. Journal style has been followed and dataset identifiers including DOIs have been expressed as full URLs.

Declarations

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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