



Plot level nitrogen stock is enhanced by a young agroforestry system in a mediterranean environment

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Abstract A 2-years assessment of nitrogen (N) stocks, tree N safety nets (from a ^{15}N labeling experiment), and biological N fixation was conducted in a Mediterranean agroforestry system in southern France. The study aimed to quantify N retention in agroforestry. The study area is characterized by a skeletal rhodic luvisol soil, a mean annual temperature of 15.5 °C, and an average annual precipitation of 556 mm. N and ^{15}N were quantified across all system components, which are N-fixing black locust trees, crops, weed plants, understory vegetation strips (UVS), rhizospheric soil, and soil microbial biomass.

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A split-plot experimental design was used, with agroforestry (AF) and monoculture (MC) plots. ^{15}N labeling was applied to track N fluxes and determine tree nutrient absorption over time. We hypothesized that tree root growth and nutrient leaching would enhance the safety-net effect, improving N retention. Results showed that crops in AF had lower N stocks (13–30% less than MC), but tree and UVS contributions compensated for the deficit, leading to a 62% increase in total plot-level N stock in 2022. The Relative Nitrogen Content was 0.97 in 2021 and 1.63 in 2022. While no ^{15}N was detected in trees in the first year (70 days after labeling), 2% of applied ^{15}N was recovered in trees 14 months post-labeling. % of N derived from atmospheric N_2 (%Nd_fa) ranged from 52 to 68%, with trees fixing 14–18 kg N ha⁻¹. We confirmed that agroforestry enhances N retention, but further research is needed to quantify leaching and gaseous losses of nitrogen.

Keywords Black locust · Safety net · ^{15}N · %Nd_fa · Biological fixation · Microbial biomass

Introduction

Agroforestry systems, which integrate trees into agricultural landscapes, offer numerous ecological and agronomic benefits. These include enhancing soil fertility, promoting biodiversity, improving carbon sequestration, and bolstering resilience to climate

change (Jose 2009; Cardinael et al. 2020; Veldkamp et al. 2023). One of the key advantages of agroforestry systems is their potential to maintain soil fertility and increase nutrient retention in the soil (Ilany et al. 2010; Elrys et al. 2023). For instance, the incorporation of trees with their understory vegetation (UVS) in agroforestry systems helps to prevent leaching of valuable nutrients by intercepting and utilizing them for tree growth, and improves their recycling and subsequent availability for the crop through litter fall, which could have positive effects on overall agroecosystem functioning (Rowe et al. 1999; Nair et al. 2007; Jose et al. 2009; Bergeron et al. 2011; Tully et al. 2012; Mahmud et al. 2024). This capacity of nutrient retention limiting nutrient lixiviation has been labelled “the safety net hypothesis” (van Noordwijk et al. 2015). However, despite widespread acknowledgment of these benefits, direct experimental evidence testing this hypothesis and quantifying nitrogen fluxes and stocks in agroforestry systems remains limited (Bergeron et al. 2011; Cannavo et al. 2013; Boinot et al. 2019; Rosati et al. 2021; D’Hervilly et al. 2020; Mahmud et al. 2024). This raises questions about the validity of this hypothesis under a wide range of growing conditions, particularly when tree plantations are young and/or when the cropping systems are high-input and planted with nitrogen fixing trees.

Another challenge in understanding the nitrogen functioning of agroforestry system is to assess the impact of the use of N_2 fixing species in the balance of crop systems. Indeed, nitrogen-fixing trees are frequently grown to counterbalance substantial nitrogen loss caused by crop harvesting and to bring natural nitrogen into the soil without adding expensive artificial fertilizer (Nygren et al. 2000; Baier 2023). Quantifying these atmospheric inputs would make it possible to better manage fertilizer inputs. Stable isotopes of nitrogen (^{15}N) are commonly employed to measure atmospheric nitrogen fixation by legumes, which hinges on the differences in isotopic composition between nitrogen available in the soil and atmospheric nitrogen (Rennie et al. 1978). Such differences may arise from the natural enrichment of ^{15}N in soil nitrogen compared to the atmosphere (Amarger et al. 1979; Ledgard et al. 1985; Barthes et al. 1995), or from the controlled addition of ^{15}N -enriched fertilizer or organic material labeled with ^{15}N (Legg and Sloger 1975; Edmeades and Goh 1978; Phillips and Bennett 1978). Marron et al. (2018)

proposed a method to quantify the proportion of nitrogen coming from the atmosphere in *Robinia pseudoacacia*. To our knowledge, this method has never been used to quantify nitrogen inputs of atmospheric origin in agroforestry systems including this tree.

This paper addresses these gaps by conducting a comprehensive assessment of the nitrogen stock and nitrogen safety net (via ^{15}N labelling) over a 2-years period (2021 and 2022) in a Mediterranean agroforestry system. The assessment covers all components of the system, including a nitrogen-fixing tree, the arable crop, weed plants in 2022, the UVS, the bulk soil, and soil microbial biomass. The quantity of nitrogen coming from the atmosphere through the biological nitrogen fixation was also evaluated at plot level. Specifically, we seek to answer the following research questions:

- How does agroforestry influence nitrogen stocks at the plot level compared to monoculture?
- Do black locust trees capture and retain nitrogen over time, acting as a nutrient safety net?
- What proportion of nitrogen in black locust trees is derived from atmospheric N_2 fixation?

What role does the understory vegetation strip (UVS) play in nitrogen cycling in the agroforestry system? We hypothesize that:

- A. Trees will absorb ^{15}N over time (14 months after labelling), due to increased root growth and nutrient leaching compared to the initial labeling.
- B. Agroforestry will store more nitrogen at the plot level than monoculture, compensating for crop nitrogen deficits through tree and UVS contributions.
- C. Soil microbial biomass (BM) will play a significant role in nitrogen retention within the system.
- D. The UVS will serve as an effective reference for estimating biological nitrogen fixation in black locust trees.

Material and methods

Study area

The study was conducted at the INRAE Instrumented Agroforestry Site (DIAMs), located at 43.612° N, 3.976° E, 10 km south of Montpellier, France. The

region experiences a Mediterranean climate, with a mean annual temperature of 15.5 °C and an average annual precipitation of 556 mm (Mauguio Station data, 2012–2021). The soil at the study site is classified as Skeletic Rhodic Luvisol (IUSS Working Group WRB 2014), characterized by high proportions of stones (up to 60%) and a pronounced red color. The soil profile consists of several layers: Ap1 (0–20 cm), a plow layer with a lumpy texture; Ap2 (20–50 cm), a second plow layer; Bt1 (50–80 cm), an illuvial layer with lattice clays and stones; Bt2 (80–125 cm), an illuvial layer with lattice clays without stones; and IICk (125+ cm), bedrock with calcium carbonates (Siegwart et al. 2023). The stone content of up to 60% is present throughout the profile, with soil texture transitioning from loamy in the upper layers to clay-rich in the illuvial horizons. The agroforestry system (AF) was established in 2017, consisting of nitrogen-fixing black locust (*Robinia pseudoacacia*) trees integrated with annual crops. The monoculture (MC) consisted only of annual crops.

Experimental design and crop management

The study followed a split-plot experimental design (see Fig. 1), with two treatments: agroforestry (AF) and monoculture (MC), each replicated twice (two blocks). In AF plots, trees were planted in rows with 2 m intra-row spacing and 17 m inter-row spacing, resulting in a density of 294 trees ha⁻¹. Between tree rows, an understory vegetation strip (UVS) was maintained. MC plots had no trees or UVS.

In the first year of the study (2021), barley (*Hordeum vulgare*) was sown between January 21st and 22nd. The fertilizer used was Smart N 46, a controlled-release urea fertilizer treated with NBPT urease inhibitor (Cantarella et al. 2018). Fertilizer was applied in two doses: the first dose, providing 40 kg N ha⁻¹, was applied on March 4th, at a rate of 87 kg ha⁻¹. The second dose, providing 60 kg N ha⁻¹, was applied on April 24th, resulting in a total nitrogen application of 100 kg N ha⁻¹ for the growing season. The use of controlled-release fertilizer was chosen to minimize nitrogen volatilization and optimize nitrogen use efficiency, promoting better nitrogen retention and reducing losses to the environment. This fertilizer rate was based on regional agronomic recommendations for barley. The barley crop was harvested between June 30th and July 2nd, 2021.

In 2022, pea (*Pisum sativum*) was sown on December 20th, 2021 in both the agroforestry (AF) and monoculture (MC) systems. Since peas are capable of fixing nitrogen naturally, no fertilizer was applied in the second growing season. The pea crop was harvested on June 9th, 2022.

Barley was grown in both agroforestry (AF) and monoculture (MC) systems in 2021, with the same amount and type of fertilizer applied to both systems to ensure consistent nitrogen availability across treatments. In the agroforestry system, barley was grown alongside black locust trees and understory vegetation strips (UVS), while in the monoculture system, barley was grown without trees or UVS.

¹⁵N labelling and sampling

To track nitrogen fluxes, ¹⁵N labeling was conducted in April 2021, coinciding with the second fertilizer application. A ¹⁵N-enriched urea solution (98% ¹⁵N, 4.6 kg ¹⁵N ha⁻¹) was applied to a 1 m × 1.5 m quadrat in both AF and MC plots. Sampling occurred in April 2021 (pre-labeling), June 2021 (harvest), and June 2022 (one year post-labeling), targeting trees (leaves, branches, roots), soil, UVS, and crops.

For crop roots, ten plants per quadrat were uprooted to 20 cm depth, and root density in the top 15 cm was extrapolated using a 15 cm × 8 cm soil auger. Tree roots were sampled by excavating soil around the trunk base. Soil samples were collected from the UVS quadrat, AF crop areas, and MC plots using a manual soil auger. All samples were oven-dried (60 °C) and ground for nitrogen and ¹⁵N analysis.

Soil microbial biomass assessment and ¹⁵N recovery

Soil microbial nitrogen and carbon content were assessed using the chloroform fumigation-extraction method. Two 5 g soil subsamples (sieved to 1 mm) were prepared: one underwent 24-h chloroform fumigation, while the other remained untreated. Extraction was performed using 20 ml of 0.5 mol L⁻¹ K₂SO₄ (for elemental analysis) and 20 ml of 0.03 mol L⁻¹ K₂SO₄ (for ¹⁵N determination) under 250 rpm shaking for 30 min. Extracts were filtered (1.2 µm GFC Whatman) and analyzed using a C and N analyzer (TOC TNM-1, Shimadzu, France). Freeze-dried samples were used for ¹⁵N analysis via an isotopic ratio mass

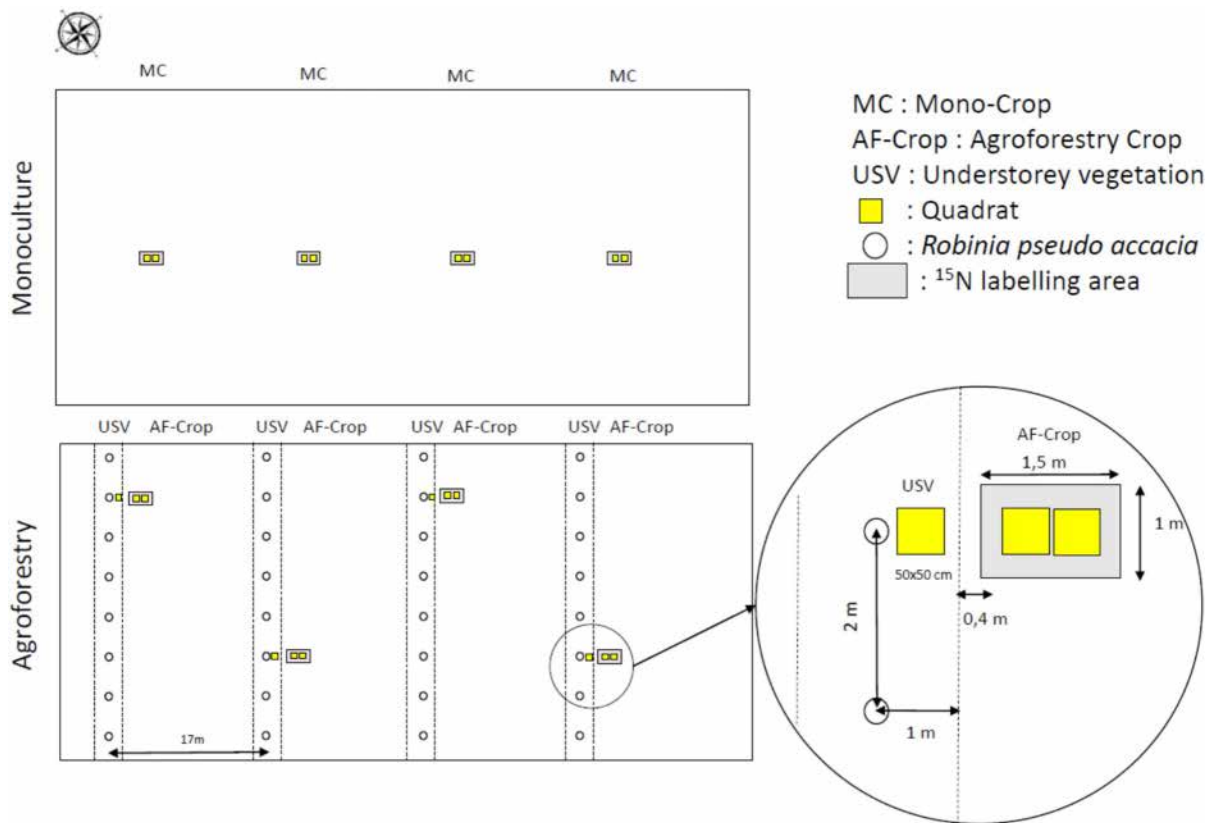


Fig. 1 Schematic of the experimental design (adapted from Mahmud et al. 2024) agroforestry (AF) and monoculture (MC) plots. In AF plots, four tree lines served as replicates, with one tree per line selected for its uniform diameter and height to act as the focal point for sampling trees, crops, soil, and understorey vegetation strips (UVS) as well as for ¹⁵N crop soil labeling. In 2021, the average tree diameter at breast height (DBH) was 4.15 ± 1.28 cm and height 3.31 ± 0.69 m, increasing in

2022 to 5.13 ± 1.44 cm and 3.76 ± 0.89 m. Crop soil labeling areas in AF plots were positioned 1.4 m perpendicular to the tree trunks. In MC plots, four sampling and labeling areas per block were aligned along an imaginary straight line and spaced 17 m apart. A 1 m × 1.5 m quadrat was used to define the ¹⁵N labeling area, within which smaller 0.5 m × 0.5 m quadrats were later employed to minimize border effects during crop sampling

spectrometer (FLASH 2000 HT/IRMS, Thermo Scientific).

Microbial biomass carbon (C-MB) and nitrogen (N-MB) were calculated using the formula:

$$C - MB = [(C \text{ in fumigated soil}) - (C \text{ in non - fumigated soil})] / 0.45.$$

$$N - MB = [(N \text{ in fumigated soil}) - (N \text{ in non - fumigated soil})] / 0.54.$$

The ¹⁵N concentrations were determined in a parallel manner from the fumigated and non-fumigated soil extracts.

The quantity of ¹⁵N from the labelled solution that was recovered in the different compartments was calculated using the following formula:

$$Recovered^{15}N = Atom\%^{15}N_{excess\ compartment} \times N\%_{compartment} \times total\ biomass\ of\ compartment.$$

$$With \quad Atom\%^{15}N_{excess\ compartment} = Atom\%^{15}N_{compartment\ after\ labelling} - Atom\%^{15}N_{compartment\ natural\ abundance}$$

N natural abundance was estimated from pre-labeling samples (April 2021) and from 6 m away from the labeling area (2022). For the 2021 spike, a reference value of 0.36 was used (Deléens et al. 1997).

Biomass metrics and nitrogen quantification

Microbial biomass C, N, and ¹⁵N ($g\ m^{-2}$ or $mg\ m^{-2}$) were calculated by extrapolating values from grams

of soil to a 1 m² area (15 cm depth). Given a mean dry soil weight of 354 g per corer sample (15 cm × 8 cm), microbial biomass values (μg g⁻¹) were multiplied by 70.56 to convert to mg m⁻².

For N and ¹⁵N determination, tree branches, leaves, roots, crop spikes, and UVS samples were pre-ground to 80 μm using a Mixer Mill MM301 (Retsch), weighed in tin capsules (D1006, Elemental Microanalysis), and analyzed using a C and N analyzer (TNM-1, Shimadzu, France) and an isotopic mass spectrometer (FLASH 2000 HT/IRMS, Thermo Scientific, INRAE, Versailles).

Tree nitrogen content was estimated from compartment biomasses (leaves, branches, trunk, stump, and roots) using allometric equations. The N content in old branches was used to estimate nitrogen in the trunk, while fine root N content was used to quantify the entire belowground nitrogen pool.

Estimation of biological nitrogen fixation (%Nd_{fa}) in *Robinia pseudoacacia*

The ¹⁵N natural abundance technique is the most commonly used method for estimating biological nitrogen fixation. It involves determining the isotopic δ¹⁵N values of three sources: (i) the legume of interest, (ii) a non-N₂-fixing reference plant growing within the same field as the aforementioned legume, and (iii) the isotopic δ¹⁵N value of the legume of interest when relying solely on biological nitrogen fixation (BNF) as the nitrogen source for growth, which provides what is known as the "B value." This B value corrects for any isotopic discrimination during the uptake and redistribution of symbiotically fixed nitrogen (Marron et al. 2018).

In a study by Marron et al. (2018), which aimed to characterize the nitrogen fixation potential of black locust in a plantation using isotopic methods, B-values for black locust in France were estimated. They employed two methods for estimation: (i) by growing trees on an N-free medium in controlled conditions (referred to as "Blab"), and (ii) by aligning %Nd_{fa} (percentage of nitrogen derived from the atmosphere) calculated with the natural abundance method to that calculated with the ¹⁵N dilution method in the field (referred to as "Bfield"). Both methods yielded consistent estimates of the B value, ranging between - 1.4 ‰ and - 3.2 ‰. In our current study, we utilized the B-values estimated by Marron et al. (2018)

for assessing nitrogen fixation by black locust in our field (i.e. - 3.2, - 2.3, - 1.4, and 0).

Some key assumptions that underpin the process of estimating %Nd_{fa} in black locust trees considered in this paper are as follows:

1. The natural abundance method was selected as the most suitable approach for quantifying Nd_{fa} in the trees. It was assumed that the targeted black locust trees do not extend their roots into the crop area, thus mitigating any potential impact of nitrogen derived from fertilizer applications to the surrounding soil's ¹⁵N content. Prior investigations conducted at the same site (Siegwart et al. 2023 through root mapping; Mahmud et al. 2024 through ¹⁵N labeling) have demonstrated this, revealing the absence of applied ¹⁵N in both the trees and the grass strip that accompanies the crop in 2021.
2. For our study, we opted to use leaves from the UVS (located at the base of the target tree) as the reference plant for tree %Nd_{fa} estimation, positing that they engage with the same soil zone as the black locust tree. In alley systems of agroforestry, a cereal, weed, broadleaf or grass can be feasibly utilized as a non-fixing reference even if root depth disparities are present compared to the fixing species (Vallis et al. 1967; Rerkasem et al. 1988; Schwenke et al. 1998; Unkovich et al. 1994, 2008).

Since leaves generally act as the primary sink for recently fixed N, we used leaves to assess the symbiotic N₂ fixation by black locust trees. δ¹⁵N values extracted from the sampled leaves of black locust and the UVS were subjected to analysis. The percentage of nitrogen derived from the atmosphere (%Nd_{fa}) was estimated using the following equation as described by (Unkovich et al. 2008; Diatta et al. 2020).

$$\%Nd_{fa} = 100 \times (\delta^{15}N_{ref} - \delta^{15}N_{fixing\ plant}) / (\delta^{15}N_{ref} - B)$$

In the above equation,

δ¹⁵N_{ref} represents the δ¹⁵N level detected in the leaves of the reference plant growing in the same soil and time frame as the black locust (UVS). δ¹⁵N_{fixing plant} is the ¹⁵N abundance of the black locust. B represents the ¹⁵N abundance (‰) of the black locust, originating solely from N₂ fixation. The B values

used in this study were specifically adapted from Marron et al. (2018) as -3.2 , -2.3 , -1.4 , and 0 for black locust at 23, 28, 40, and 52 months in France.

N stock determination and relative nitrogen content (RNC)

The estimation of total nitrogen stock was conducted at both the component and plot levels within the AF and MC systems. This involved a comprehensive assessment of the nitrogen stock across various compartments, including crop biomass, rhizospheric soil, soil microbial biomass, the UVS, and the trees. Subsequently, these estimations were scaled up to provide an assessment at the plot level.

In both the AF and MC sites, the crop plots were standardized with dimensions of 17 m in width and 100 m in length, resulting in a total area of 1700 m². While in the MC site, the entire area was dedicated to crops, in the AF site, some portions were allocated to represent the UVS and trees. These allocated areas were 2 m in width, matching the width of the UVS, and extended 100 m in length, totalling 200m². Since the AF trees were spaced 2 m apart along the tree line, each plot within the AF system therefore contained 50 trees.

Furthermore, the Relative Nitrogen Content (RNC) in the vegetation was determined at the plot level. This ratio facilitated a comparison of nitrogen content between agroforestry and monocrop systems, offering insights into the nitrogen dynamics and efficiency of each respective system.

The RNC was calculated using the formula:

$RNC = (NAF/NMC)$, where NAF is the nitrogen quantity in agroforestry (ie the sum of trees, crop and UVS) and NMC is the nitrogen quantity in the monoculture.

Statistical analysis

Data analysis was conducted using the R program (R Core Team, 2022). To evaluate the impact of crop type (AF-crop, UVS, MC) on the measured variables, Generalized Linear Mixed Models (GLMMs) were employed, assuming a Gaussian distribution. The block variable was included as a random effect in these models.

In instances where significant effects were detected among the measured modalities, a Tukey Honestly Significant Difference (HSD) test was carried out to pinpoint specific modalities that exhibited significant differences compared to the others. The threshold for statistical significance was set at $\alpha = 5\%$.

Results

¹⁵N excess in crop, tree and soil compartments

In 2021, the AF and MC crops exhibited a higher percentage of ¹⁵N excess (ranging between 1 and 2%) compared to UVS and tree compartments, where no detectable ¹⁵N excess was observed (Fig. 2a). In 2022, the ¹⁵N excess levels were lower but remained positive across all crops (AF and MC), with a small amount of ¹⁵N detected in trees and UVS (Fig. 2b).

For soil compartments, in 2021, the percentage of ¹⁵N excess in AF and MC soils was approximately 2%, whereas in UVS soil, it was significantly lower (0.009%). By 2022, the ¹⁵N excess in AF and MC soils had decreased to 0.035%, while the UVS soil retained only 0.01%.

¹⁵N quantity in excess in crop, tree and soil compartments

In 2021, a total of 460 mg m⁻² of ¹⁵N was applied to the soil. After two months, 47% of the applied ¹⁵N was absorbed by crops in both AF and MC (Fig. 3a), while approximately 40% remained in the rhizospheric soil, with no detectable retention in BM. The remaining 13% of ¹⁵N was unaccounted for in the crops, soil, or BM.

In 2021, the ¹⁵N excess levels in MC and AF crops were similar across their respective compartments. However, in 2022, this trend reversed, with AF crops exhibiting higher ¹⁵N than MC crops. That same year, only trace amounts of ¹⁵N were detected in BM, with values of 9 ± 8 ng ¹⁵N excess per gram of dry soil in both AF and MC systems.

The primary storage compartment for ¹⁵N in 2022 was soil, where AF-crop and MC soils retained between 34.4 and 37.7 mg ¹⁵N excess m⁻², while UVS soil contained 11.7 mg ¹⁵N excess m⁻² (Fig. 3b).

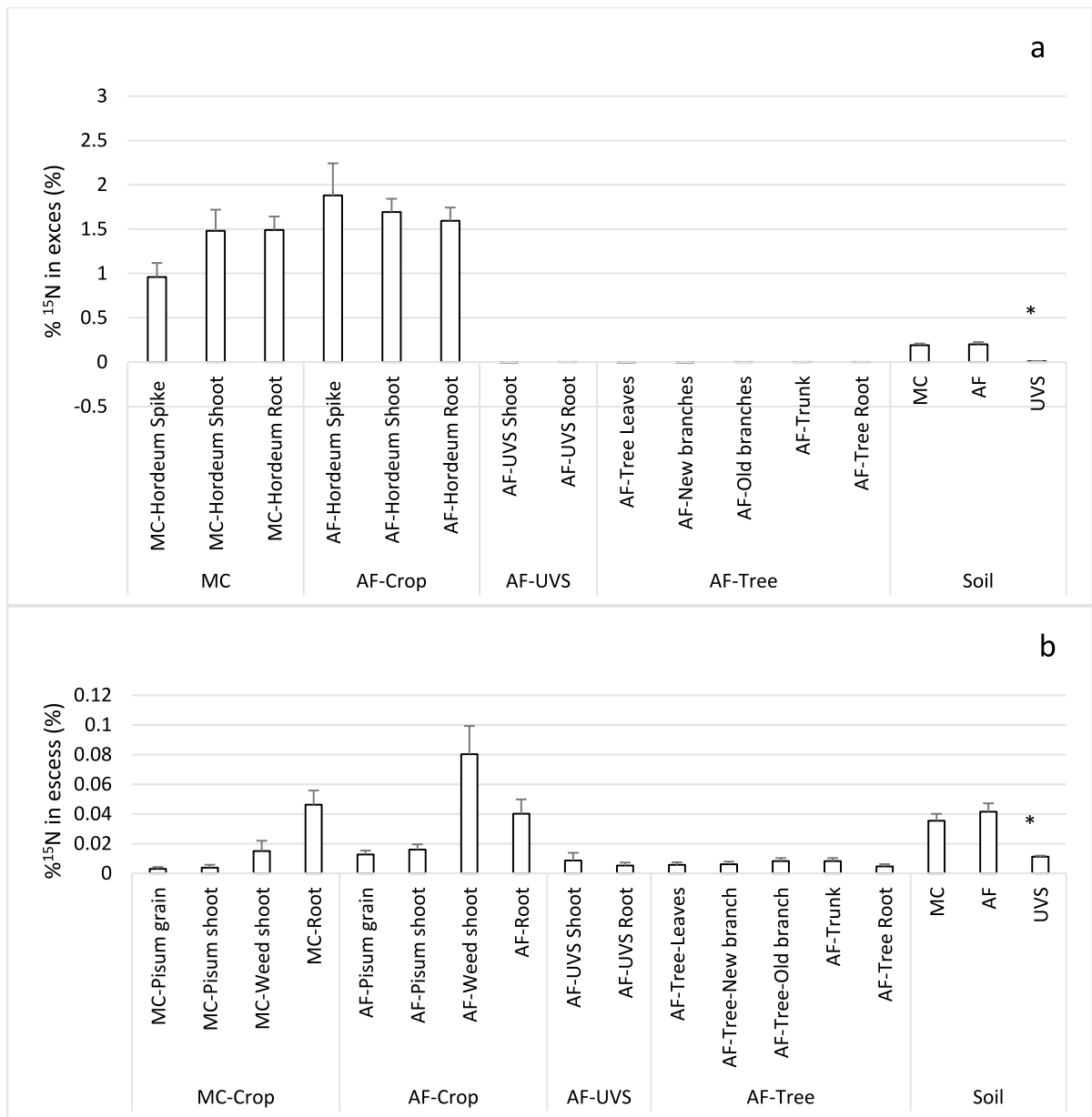


Fig. 2 Percentage of ¹⁵N in excess in different compartments of monocrop (MC), agroforestry crop (AF-Crop), understory vegetation strip (UVS) and tree (AF-tree) in 2021 (a) and 2022

(b). Values are means and standard error of eight field replicates. Error bar with * indicates a treatment that was significantly different between MC; AF and UVS in soil

In 2022, the AF-Crop, MC and tree systems had values of 0.5 mg m⁻², 2.5 mg m⁻², and 10 mg tree⁻¹ of ¹⁵N in excess respectively, confirming the uptake of ¹⁵N by the trees which is in contrast to 2021 where no ¹⁵N uptake was observed in the tree and UVS (Fig. 3b). ¹⁵N in excess was distributed across all tree compartments, including the leaf (2.3 mg tree⁻¹), old

branch (0.6 mg tree⁻¹), new branch (0.1 mg tree⁻¹), trunk (3.0 mg tree⁻¹), and roots (3.8 mg tree⁻¹) (Fig. 3b). UVS also had 0.6 mg of ¹⁵N m⁻² in the root and shoots.

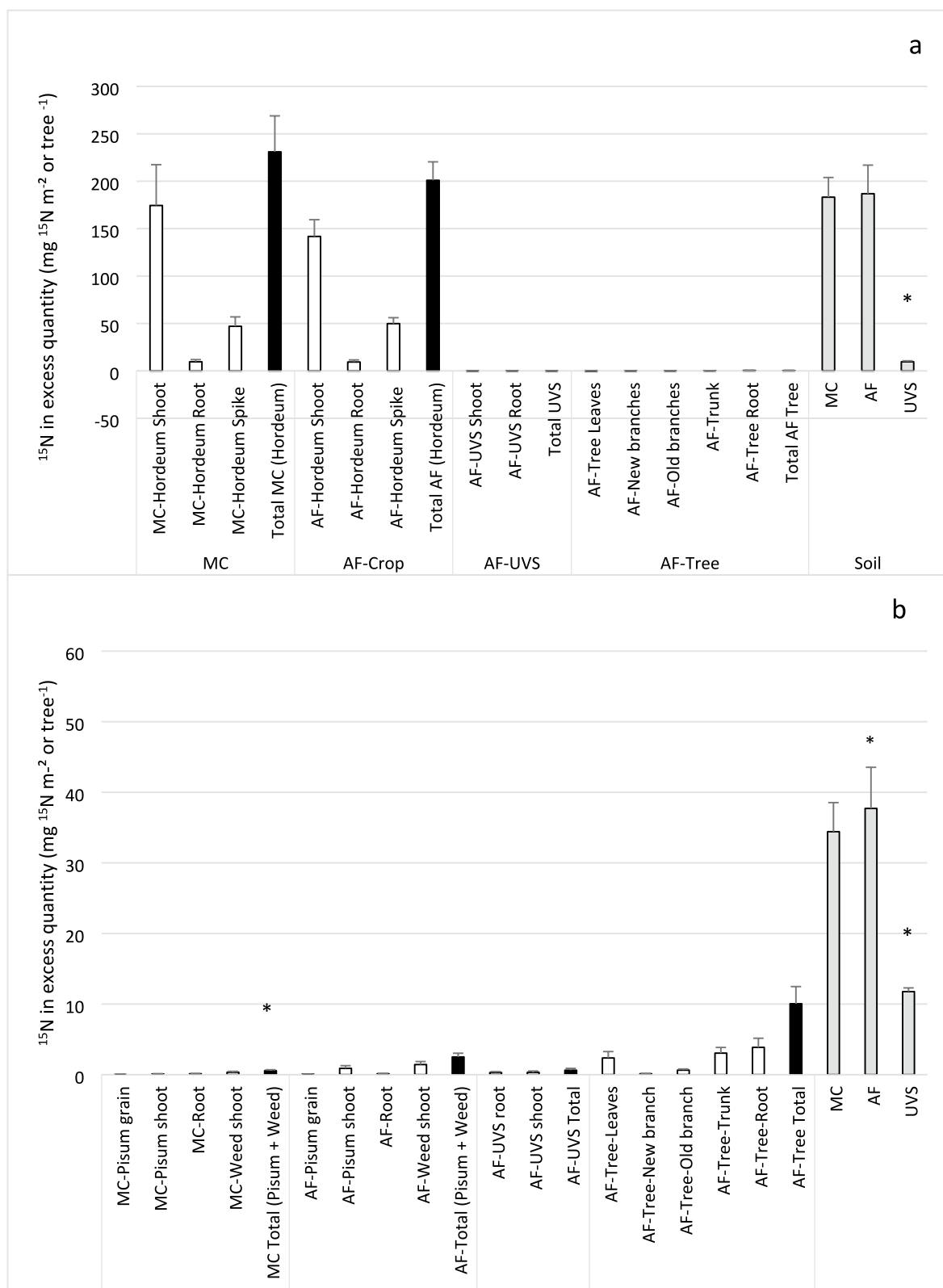


Fig. 3 Quantity of ^{15}N in excess in different compartments of monocrop (MC), agroforestry crop (AF-Crop), understory vegetation strip (UVS), tree (AF-tree) and rhizospheric soil in 2021 (a) and 2022 (b). The data concerning MC and AF-Crop in 2021 (a comes from Mahmud et al. 2022). Values are means and standard error of eight field replicates. Error bar with * indicates treatment that was significantly different between MC and AF in crop and between MC; AF and UVS in soil

Nitrogen quantity in crop, tree and soil compartments

In 2021, the tree system stands out with the highest total nitrogen content ($p=0.001$) ($136 \pm 28 \text{ gN tree}^{-1}$), driven by significant amounts in the leaf ($36 \pm 7 \text{ gN tree}^{-1}$) and root ($53 \pm 21 \text{ gN tree}^{-1}$) compartments (Fig. 4a). Both MC ($16 \pm 3 \text{ gN m}^{-2}$) and AF ($12 \pm 4 \text{ gN m}^{-2}$) show relatively higher total nitrogen quantities compared to UVS with $4.8 \pm 3 \text{ gN m}^{-2}$, which has the lowest total nitrogen content, with less nitrogen in both shoot ($4 \pm 3 \text{ gN m}^{-2}$) and root compartments ($0.44 \pm 0.3 \text{ gN m}^{-2}$).

In 2022, nitrogen distribution in the AF and MC systems included contributions from pea and weeds. In the MC system, pea grains contained $0.40 \pm 0.2 \text{ g N m}^{-2}$, while shoots held approximately $4 \pm 3 \text{ g N m}^{-2}$, and roots contained $0.24 \pm 0.15 \text{ g N m}^{-2}$. Additionally, weed shoots in MC accumulated $3 \pm 4 \text{ g N m}^{-2}$. In the AF system, nitrogen content was slightly higher, with pea grains, shoots, and roots containing 0.48, 4.88, and 0.36 g N m^{-2} , respectively. The weed shoot in AF stored approximately 2.08 g N m^{-2} . Consistent with 2021, the tree system remained the largest nitrogen reservoir, with a total nitrogen content of $169.89 \text{ g N tree}^{-1}$, primarily distributed in the leaf ($43.95 \text{ g N tree}^{-1}$) and root (81.75 g N m^{-2}) compartments. Both MC (7.9 g N m^{-2}) and AF (7.8 g N m^{-2}) systems exhibited lower total nitrogen quantities compared to the UVS (8.63 g N m^{-2}) and trees (Fig. 3b).

The soil nitrogen content remained stable across both years, with no significant differences between AF and MC systems.

Relative nitrogen content

Table 1 presents the nitrogen content at the plot level. In the MC, the plot consisted of 1 hectare of crops, whereas in the AF, the plot configuration included a 15 m-wide alley crop and a 2 m-wide understory vegetation strip (UVS), scaled up to 1 hectare. The addition of trees and UVS in AF significantly influenced

total nitrogen content compared to MC. In 2021, the MC crop stored 159 kg N ha^{-1} , whereas the AF crop stored 108 kg N ha^{-1} , showing that MC had over 50% more nitrogen than AF ($p=0.007$), primarily due to differences in crop biomass per m^2 (Mahmud et al. 2024). However, with the inclusion of UVS and trees, the total plant nitrogen content in AF increased to 154 kg N ha^{-1} , closely matching 159 kg N ha^{-1} in MC, resulting in an RNC of 0.97—nearly compensating for the crop biomass difference.

In the soil compartment, in 2021, the MC crop-soil contained $952.9 \text{ kg N ha}^{-1}$, while the AF crop-soil contained $818.3 \text{ kg N ha}^{-1}$, though the difference was not statistically significant. Additionally, the UVS-soil in AF contributed 116 kg N ha^{-1} . At the plot level, the total soil nitrogen stock was $952.9 \text{ kg N ha}^{-1}$ in MC and $934.3 \text{ kg N ha}^{-1}$ in AF, showing only a minor difference between the two systems.

In 2022, with the integration of UVS and trees into the agroforestry system (barley + pea + Weed + UVS + Tree), the total plant nitrogen content increased to 129 kg N ha^{-1} . This was significantly higher than the MC system, which, without UVS and trees, stored only $79.2 \text{ kg N ha}^{-1}$. Consequently, the Relative Nitrogen Content increased to 1.63, indicating that the agroforestry system stored 63% more nitrogen in plant biomass compared to what a monoculture system would achieve under similar land allocation. The nitrogen stored in microbial biomass accounted for 1–10% of total soil nitrogen content. In both 2021 and 2022, the majority of MB nitrogen was found in the crop soil, ranging between 5 and 13 kg N ha^{-1} in 2021 and 6–9 kg N ha^{-1} in 2022. The UVS MB contribution remained below 1.5 kg N ha^{-1} in both years.

Percentage nitrogen derived from the atmosphere (%Nd_{fa})

As shown in Table 2, the percentage of nitrogen derived from atmospheric N_2 (%Nd_{fa}) in black locust trees ranged from 52 to 68%, depending on the B-value used. The variation in B-value did not significantly affect the Nd_{fa} estimation ($p=0.065$). The biological nitrogen fixation rate of black locust in the agroforestry system ($294 \text{ trees ha}^{-1}$) was estimated at 14–18 kg N ha^{-1} .

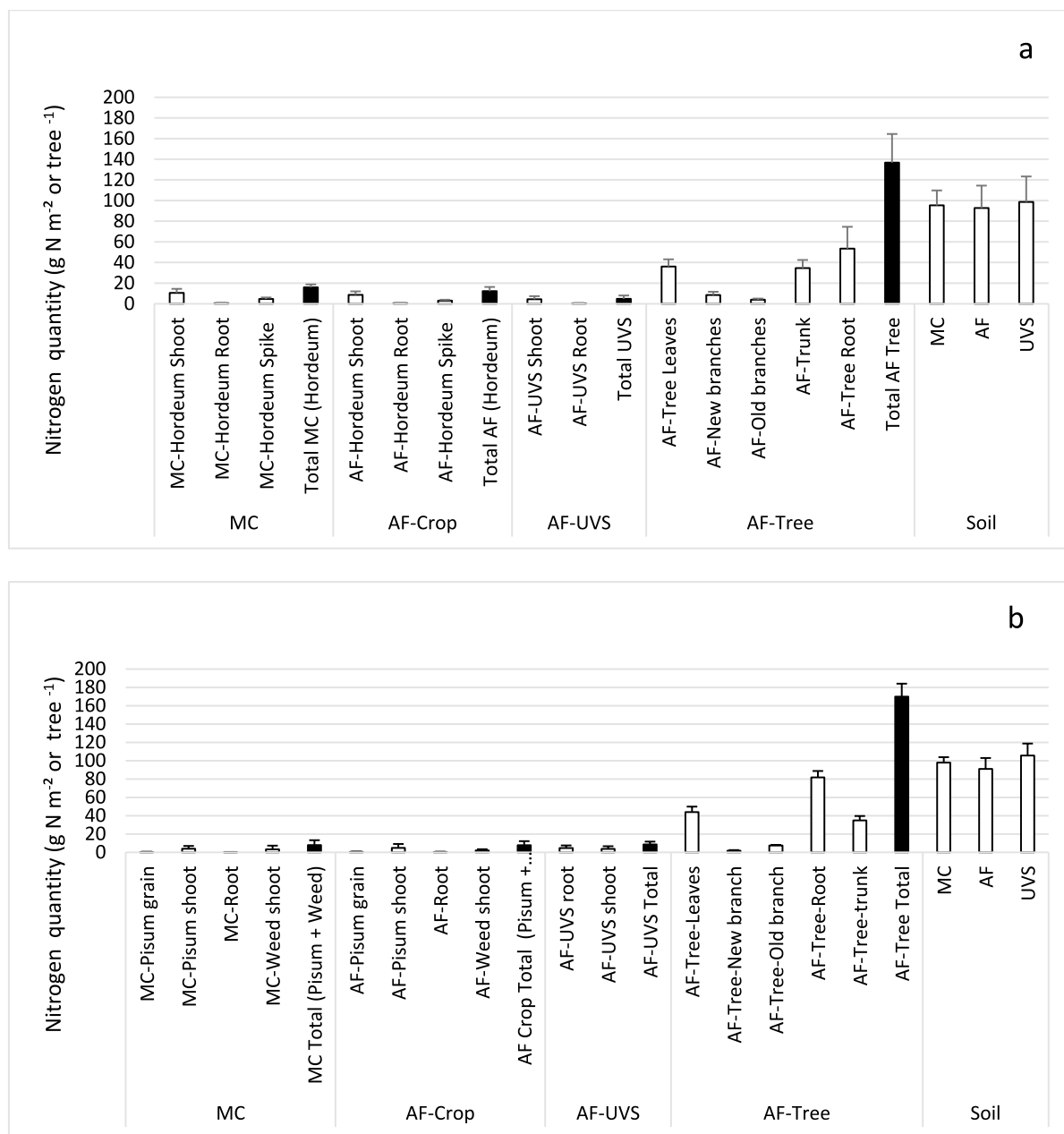


Fig. 4 Quantity of nitrogen in different compartments of monocrop (MC), agroforestry crop (AF-Crop), understory vegetation strip (UVS), tree (AF-tree) and rhizospheric soil in

2021 (**a**) and 2022 (**b**) expressed in g N m⁻² or in g N tree⁻¹. Values are means and standard error of eight field replicates

Discussion

Tree nutrient safety net and ¹⁵N recovery

In 2021, no ¹⁵N uptake was detected in trees, likely due to limited nitrogen leaching, slow root growth, and substantial retention of ¹⁵N in the soil (evident from high amount of ¹⁵N obtained in the soil in the first year). Low cumulative rainfall (51.5 mm) and the use of urea with a urease inhibitor which moderates

Table 1 Nitrogen stocks at the plot level in monoculture (MC) and agroforestry (AF) systems across different compartments, including the crop, understory vegetation strip (UVS), and trees

The Relative Nitrogen Content (RNC) is calculated from the total biomass, i.e., the nitrogen in the crop (for MC) and the sum of nitrogen in the crop, UVS, and trees (for AF). In 2021, the MC plot consisted of barley, while the AF plot included barley, trees, and UVS. In 2022, the MC plot comprised pea and weeds, while the AF plot included pea, weeds, trees, and UVS. Values represent the means from eight field replicates, with standard deviations provided in parentheses. Superscript letters indicate significant differences between AF and MC

Year	Compartment	N quantity in MC (kg N ha ⁻¹)	N quantity in AF (kg N ha ⁻¹)	RNC
2021	Plant			
	Crop	159.1 (28.5) a	108.2 (35.6) b	0.97
	UVS	–	5.6 (3.8)	
	Tree	–	40.2 (8.1)	
	Plot Plant Biomass	159.1 (28.5)	154 (36.7)	
	BM			
	Crop-BM	13.4 (5.4) a	5.5 (2.7) b	
	UVS-BM	–	1 (0.3)	
	Plot BM	13.4 (5.4) a	6.6 (2.7) b	
	Soil			
	Crop-Soil	952.9 (144)	818.3 (191.4)	
	UVS-Soil	–	116.0 (28.9)	
	Plot Soil	952.9 (144)	934.3 (193.6)	
	Plot			
2022	Plant Biomass + BM + Soil	1125.5 (29.5)	1095.12 (197.6)	
	Plant			1.63
	Crop + weed	79.2 (2.7)	68.8 (39)	
	UVS	10.1 (3.6)		
	Tree	50 (4.1)		
	Plot Plant Biomass	79.2 (53.2) a	129 (39.4) b	
	BM			
	Crop-BM	6.2 (2.1)	9.4 (5.3)	
	UVS-BM	1.3 (0.3)		
	Plot BM	6.2 (2.1) a	10.7 (5.4) b	
	Soil			
	Crop-Soil	978.9 (59.5)	803.2 (105)	
	UVS-Soil	124.2 (15.4)		
	Plot Soil	978.9 (59.5)	927.4 (106.1)	
	Plot			
	Plant Biomass + BM + Soil	1064.4 (80)	1067.2 (113.4)	

Table 2 Percentage of Nitrogen derived from the atmosphere (%Ndfa) in *Robinia pseudoacacia* with UVS herbaceous plants as the reference plant

B Value	Ndfa (%)	Fixation rate (kg N ha ⁻¹)
– 3.2	52 (6)a	14 (3)a
– 2.3	56 (6)a	15 (3)a
– 1.4	60 (7)a	16 (4)a
0	68 (8)a	18 (4)a

Values are means and standard deviations of eight field replicates obtained in 2021. Values inside the parentheses are standard deviations of the means. B-values were adapted from Marron et al. 2018

ammonium release may have further reduced nitrogen leaching. In contrast, by 2022, trees absorbed 2% of the applied ¹⁵N, confirming their capacity to capture residual nitrogen over time. This result agrees with our Hypothesis A, which proposed that trees would absorb ¹⁵N over time due to increased root growth and nutrient leaching. Some studies also show that deep-rooted trees can intercept leached nutrients and reduce nitrogen losses (Zhang et al. 2019; Allen et al. 2004). Although the presence of ¹⁵N in trees and UVS in the second year supports the nutrient safety-net hypothesis, further studies are needed to quantify direct nitrogen leaching and gaseous losses.

The absence of detectable ^{15}N in microbial biomass in both AF and MC systems in 2021 contradicts our Hypothesis C, which anticipated a significant role of microbial biomass in nitrogen retention. Recent research, however, indicated that subsoil microorganisms which were not sampled in our topsoil (0–15 cm) may significantly contribute to nutrient retention in agroforestry systems (Beule et al. 2022). In contrast, studies examining microbial communities down to 60 cm have reported a strong response to tree root-derived inputs, suggesting that subsoil microbes play a more pronounced role than those in the topsoil (Zhang et al. 2017; Sosa-Hernández et al. 2018; Naylor et al. 2022). Thus, further investigation into subsoil microbial communities is essential to fully understand their contribution to the nutrient safety-net function in Mediterranean agroforestry systems.

Nitrogen stock and agroforestry benefits

In our study, nitrogen stored in crop biomass and soil was higher in monoculture than in agroforestry, likely due to reduced crop biomass in the latter. However, the inclusion of trees and UVS in agroforestry significantly boosted total plant nitrogen stocks at the plot level—nearly compensating for the crop deficit in 2021 and increasing plot-level nitrogen by 63% in 2022 (RNC rose from 0.97 to 1.63). This supports Hypothesis B, which predicted that agroforestry would store more nitrogen at the plot level than monoculture due to tree and UVS contributions. Although soil nitrogen measured at 15 cm depth was similar between systems, this may reflect the young age of the agroforestry site and the shallow sampling depth, as previous research indicates that soil nitrogen stocks in agroforestry increase with depth (Sharrow and Ismail 2004).

Moreover, a meta-analysis of 48 studies found that agroforestry systems have about 13% higher soil nitrogen stocks than monocultures (Muchane et al. 2020), and other studies have reported 33% greater nitrogen storage in agroforestry soils (Li et al. 2020) and nearly double the aboveground nitrogen storage compared to monocultures, with trees contributing an additional 33 kg N ha⁻¹ (Sharrow and Ismail 2004).

Biological nitrogen fixation in black locust

We estimated biological nitrogen fixation (%Nd_{fa}) in black locust using the ^{15}N natural abundance method, which compares $\delta^{15}\text{N}$ values of the fixing species, a non-fixing reference, and plants relying solely on N₂ fixation (providing the “B value” to correct for isotopic discrimination) (Marron et al. 2018). Our four-year-old trees showed %Nd_{fa} values of 52–68%, lower than previously reported (Marron et al. 2018), likely due to nitrogen fertilization inhibiting fixation (Uddin et al. 2008; Cusack et al. 2009; Unkovich et al. 2010). Despite this, trees fixed 14–18 kg N ha⁻¹, representing an appreciable nitrogen input. These results confirmed Hypothesis D, which proposed that the UVS would serve as an effective reference for estimating biological nitrogen fixation in black locust trees. Variability in %Nd_{fa} among studies is influenced by factors such as seasonal changes, CO₂ concentration, symbiotic microbes, soil properties, and seed provenance (Boring et al. 1984; Feng et al. 2004; Tian et al. 2003; Noh et al. 2010; Berthold 2005; Moshki and Lamersdorf 2011; Mantovani et al. 2015; Veste et al. 2012), as well as differences in rhizobial strains (Steele et al. 1983; Yoneyama et al. 1986; Zapata et al. 1987; Guimarães et al. 2008; Santachiara et al. 2017; van Vugt et al. 2018).

Understanding the behavior of *Robinia pseudoacacia* and other agroforestry components across different soil types and climatic conditions is important for applying our findings to diverse agricultural settings.

While our study was conducted in a Mediterranean climate with skeletal rhodic luvisol soil (high stone content and moderate fertility), regional studies have reported varying nitrogen fixation rates and retention efficiencies in agroforestry systems depending on soil texture and rainfall patterns (Sharrow and Ismail 2004; Muchane et al. 2020). For instance, *Robinia pseudoacacia* in temperate regions with clay-rich soils has shown higher nitrogen fixation potential (up to 90 kg N ha⁻¹ year⁻¹), whereas sandy soils in drier climates may limit root expansion and nitrogen uptake (Moshki and Lamersdorf 2011). Similarly, UVS composition varies regionally, with clover-based UVS in Northern Europe enhancing nitrogen retention, while grass-dominated UVS in Mediterranean systems provide greater erosion control but lower nitrogen fixation (D’Hervilly et al. 2020).

Practical implications for agroforestry systems

Our findings highlighted the potential of *Robinia pseudoacacia* in agroforestry systems, but their application in real-world farming requires targeted strategies to maximize nitrogen retention and reduce synthetic fertilizer dependence. Farmers can optimize nitrogen conservation by strategically integrating nitrogen-fixing trees at appropriate densities (e.g., 294 trees ha⁻¹ as tested in this study) while maintaining understory vegetation strips, which act as nitrogen sinks (D'Hervilly et al. 2020). To further enhance nitrogen use efficiency, incorporating controlled-release fertilizers (CRFs)—which can reduce nitrogen losses by 20–50% compared to traditional fertilizers—may be beneficial, particularly when combined with biological nitrogen fixation (Tian et al. 2017; Tang et al. 2018). While CRFs tend to be more expensive, their long-term cost-effectiveness, through improved nitrogen retention and reduced application frequency, could offset initial costs (Cantarella et al. 2018). Additionally, gradual fertilizer reductions based on soil testing and adaptive management strategies could help balance productivity and sustainability (Nair et al. 2007).

Practical recommendations for monitoring and evaluation

Our study provided insights into nitrogen retention in agroforestry. However, improving future methodologies for assessing nitrogen uptake and leaching dynamics is essential. One key area for improvement is the enhanced monitoring of nitrogen leaching and gaseous nitrogen losses. Future studies should incorporate gas emission sensors (e.g., closed-chamber or automated flux measurement systems) to directly quantify nitrous oxide (N₂O) and ammonia (NH₃) emissions, which are critical components of nitrogen cycling (Tang et al. 2018). Additionally, more detailed soil sampling at different depths (e.g., 0–15 cm, 15–30 cm, and 30–60 cm) could help assess nitrogen retention beyond the topsoil and capture the role of tree roots in deeper layers (Beule et al. 2022). The use of isotopic tracers and ¹⁵N pool dilution techniques could further improve our understanding of nitrogen dynamics in agroforestry systems (Zhang et al. 2019). Monitoring nitrogen under different management conditions, such as varying tree densities,

fertilizer applications, and UVS compositions, would provide knowledge into optimizing agroforestry for sustainable nitrogen retention. Future research should also integrate lysimeter studies and soil solution sampling to track nitrogen leaching under different land-use scenarios.

Implications for climate-resilient agriculture

Enhancing nitrogen stocks through agroforestry is particularly relevant in the context of climate change and nitrogen limitation under elevated CO₂ (Finzi et al. 2006; Elrys et al. 2023). Drought stress—a hallmark of future climates—has been shown to increase nodule biomass in black locust, sustaining nitrogen fixation despite lower soil nitrogen availability (Mantovani et al. 2015), making them valuable for soil fertility management and long-term agroecosystem resilience.

Conclusion

This study demonstrated that agroforestry enhances nitrogen stocks at the plot level, with trees and UVS compensating for crop nitrogen deficits. While no ¹⁵N uptake was detected in trees in the first year, 2% of applied ¹⁵N was recovered after 14 months, confirming their potential role as a nutrient safety net. The inclusion of trees and UVS increased total nitrogen storage by 63% in 2022 compared to monoculture.

Black locust trees fixed 14–18 kg N ha⁻¹, though fertilization likely reduced nitrogen fixation efficiency. Future research should investigate nitrogen transfer from trees to crops and directly measure leaching and gaseous nitrogen losses to refine nutrient management strategies. While our findings highlighted the potential of agroforestry systems in enhancing nitrogen retention, further research is needed to assess their long-term sustainability in different environmental and agronomic contexts.

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execution. I.B. contributed to the study design, field work and data acquisition, data interpretation, supervision, and project execution. J.N. provided review and editing support as well as data acquisition. Soline Martin Blangy focused on data acquisition. S.B. contributed through conceptualization, study design, supervision, results interpretation, and review and editing. N.D. was involved in conceptualization, study design, supervision, results interpretation, and review and editing. P.L. also contributed to conceptualization, study design, supervision, results interpretation, and review and editing. G.V. handled laboratory analysis, data analysis, and results interpretation. C.M. contributed through review and editing, data acquisition, and field work. Rémi Dugue participated in field work and data acquisition. A. M. was involved in field work, data acquisition, and project execution, while M.F. contributed to data acquisition. A.M. (Anne Marmagne) was responsible for data acquisition and laboratory analysis. L.B. contributed to conceptualization, study design, field and laboratory work, data analysis and interpretation, drafting the manuscript, review, editing, project execution, and supervision.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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