

RESEARCH LETTER

Legacy effects of 41 years of tillage and fertilization on biological nitrogen fixation and nutritional status of hairy vetch in a continuous cotton system

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Abstract

Legume cover crops (CCs) rely on a dynamic contribution of soil-derived nitrogen (N) and biological nitrogen fixation (BNF) to maintain optimum N status, yet how long-term agronomic management shapes this relative contribution remains poorly understood. This study leveraged a 41-year continuous cotton (*Gossypium hirsutum* L.) system to evaluate how legacy effects of tillage and N fertilization influence BNF, legume N nutritional status, and N sources in hairy vetch (*Vicia villosa*). Using the ^{15}N natural abundance method and multivariate analyses, we found that tillage was the dominant factor affecting N dynamics: no-till (NT) plots exhibited higher N status, soil organic matter, soil-derived N, and total N uptake than conventional tillage. Fertilization effects were less pronounced but modulated BNF efficiency. However, NT alone was insufficient to overcome N limitation under unfertilized conditions, as indicated by similarly low nitrogen nutrition index values and overlapping distributions in both CT-0N and NT-0N treatments, highlighting the need for integrated management. These findings underscore how legacy effects of long-term practices shape N acquisition by legumes and support combining NT with crop rotation-specific N fertilization to optimize N inputs and sustain CC performance in low-input systems.

Plain Language Summary

This study examined how long-term farming practices affect how legume cover crops, like hairy vetch, obtain nitrogen (N) from the soil and the air through biological nitrogen fixation. Using a 41-year continuous cotton experiment, we tested how different

Abbreviations: BNF, biological nitrogen fixation; CC, cover crop; CT, conventional tillage; DM, dry matter; Ndfa, nitrogen derived from the atmosphere; NNI, nitrogen nutrition index; NT, no-till; SOM, soil organic matter.

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tillage and fertilization influence vetch N uptake and sources. We found that tillage had the greatest effect: no-till soils had more organic matter, greater total N uptake, and higher soil-derived legume biomass N compared to conventional tillage. Fertilizer history had smaller but measurable impacts on how efficiently vetch fixed N. However, no-till alone was not sufficient to prevent N limitation in legume cover crops when no fertilizer was applied. These results show that the legacy of long-term management strongly influences how legumes acquire N and highlight the importance of management to sustain cover crop performance in low-input systems.

1 | INTRODUCTION

Achieving sustainable crop production while reducing adverse environmental impacts requires the continued adoption of soil health-promoting practices. Among these practices, long-term no-tillage (NT) and strategic use of leguminous cover crops (CCs) are central to conservation agriculture, contributing to improved soil structure, enhanced organic matter accumulation, and efficient nutrient cycling (Blanco-Canqui & Ruis, 2018; Blanco-Canqui et al., 2015; Lussich et al., 2024). However, sustaining these benefits also depends on effective nutrient management, particularly nitrogen (N), which governs both soil fertility and greenhouse gas outcomes, including crop N availability, soil organic matter (SOM) dynamics, and N₂O emissions (Blanco-Canqui et al., 2015). N management remains a critical challenge in conservation agriculture systems, as it regulates soil N availability and losses, thereby influencing crop productivity, cost of production, and environmental outcomes (Dhaliwal et al., 2024; Nouri et al., 2021). In this context, biological nitrogen fixation (BNF) by leguminous CCs, such as hairy vetch (*Vicia villosa*), provides the capacity to acquire atmospheric N, potentially reducing the dependency on synthetic fertilizers and improving the N use efficiency and balance of the system (Louarn et al., 2021; Parr et al., 2011).

While the benefits of NT and leguminous CCs on soil health and cash crop performance are well documented (Blanco-Canqui et al., 2011), less is known about how their long-term legacy effects influence the N fixation, N status, and N acquisition strategies of subsequent leguminous CCs. Specifically, how these management factors influence the relative contributions of soil-derived versus atmospherically fixed N remains unclear. This gap is particularly relevant for legumes, which are often assumed to meet their N needs through BNF alone. Because BNF is energetically costly and sensitive to soil fertility and external N inputs, reduced BNF shifts plant N acquisition toward soil-derived N, with consequences for soil N depletion, fertilizer requirements by subsequent crops, and potential N losses. As a result, legumes exhibit flexible N acquisition strategies, adjusting

their reliance on soil-derived N versus atmospheric N fixation in response to soil N availability. Quantification of nitrogen derived from the atmosphere (Nd_{fa}) commonly relies on the ¹⁵N natural abundance method, which compares legume ¹⁵N signatures with co-occurring non-legume reference plants and is a well-established and recommended approach in agricultural systems (Unkovich et al., 2008).

In continuous cotton (*Gossypium hirsutum* L.) production systems characterized by low residue inputs and moderate N fertility, maintaining adequate N availability for the cash crop represents a key constraint to long-term productivity and sustainability (Nouri et al., 2019). In this context, variation in the balance between BNF and soil-derived N in legume CCs influences their net N contribution to the system, with implications for fertilizer requirements, soil N balance, and potential N losses. Moreover, few studies have used integrative, biomass-adjusted indicators such as the nitrogen nutrition index (NNI), with NNI values below one indicating N limitation (Sadras & Lemaire, 2014), to assess legume N status in long-term conservation systems. When combined with estimates of the proportion of Nd_{fa}, this approach clarifies how legumes balance soil- versus atmosphere-derived N acquisition under different fertility conditions, informing whether they function as net N contributors or rely on mining existing soil N pools. To address these knowledge gaps, this study utilizes a 41-year continuous cotton cropping system and integrates plant and soil measurements with multivariate analyses to evaluate how the implementation of long-term tillage and N fertilization practices affect BNF, legume N nutritional status, and N acquisition strategies in vetch CC under low-input systems.

2 | MATERIALS AND METHODS

2.1 | Study site and experimental design

This study was conducted in 2022 and 2024 (CCs failed to establish in 2023) within a long-term cotton cropping experiment established in 1981 at the University of Tennessee in

Jackson, Tennessee (35°37'22" N, 88°50'47" W), on a Lexington silt loam soil (fine-silty, mixed, thermic, and Ultic Hapludalfs). The experiment follows a randomized complete block design with a split-split plot arrangement of N rates, CCs, and tillage treatments, including four replications and a total of 128 experimental units (12 m × 8 m). Treatment and management practices, consistently applied to the same plots since 1981, are described in detail by Nouri et al. (2020). For this study, 32 vetch subplots were used, which included four N levels (0, 34, 67, and 101 kg N ha⁻¹) and two tillage levels (NT and conventional tillage; CT). This combination resulted in eight treatments: CT-0N, CT-34N, CT-67N, CT-101N, NT-0N, NT-34N, NT-67N, and NT-101N—with four replicates each. Fertilization and tillage treatments were applied during the preceding cotton crop, while N fixation and N status were evaluated in the subsequent vetch CC growing season, a preferred legume CC when maximizing BNF is a priority. Weather conditions during the vetch growing seasons and average aboveground biomass for each study year are summarized in Table S1.

2.2 | Plant and soil measurements

Prior to vetch termination each year in April–May, aboveground plant biomass was harvested to determine total dry matter (DM) production. Total N and natural abundance of ¹⁵N, expressed as δ¹⁵N, were analyzed in both vetch and associated non-legume reference plants (broadleaf weeds) in each plot, following recommendations by Unkovich et al. (2008).

BNF was estimated as the percentage of N derived from atmosphere (%Nd_{fa}) using the ¹⁵N natural abundance method based on the δ¹⁵N values of the legume (δ¹⁵N_{legume}), the non-fixing reference plant (δ¹⁵N_{ref}), and a published reference B-value (−0.79‰; Unkovich et al., 2008), according to Equation (1):

$$\text{Nd}_{fa} (\%) = \frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{legume}}}{\delta^{15}\text{N}_{\text{ref}} - B} \quad (1)$$

We additionally estimated total N uptake (plant N concentration × biomass), N fixed in vetch aboveground biomass (%Nd_{fa} × total N uptake), and N derived from soil (by difference between total N uptake and N fixed), all expressed in kg N Mg⁻¹ to standardize N content per unit of dry biomass.

To assess N status, we calculated the NNI, which compares actual tissue N concentration (%N) to a critical concentration (%N_c) derived from the N dilution curve for legumes (Lemaire, 1997; Lemaire et al., 1985). NNI values close to one indicate optimal N status, whereas values <1 reflect N limitation for plant growth. The NNI was calculated according to

Core Ideas

- Hairy vetch grown under conventional tillage was more limited by N than no-till, with differences widening at higher N fertilization rates.
- Atmospheric N contribution was higher under conventional tillage than no-till, with greater differences at higher fertilization rates.
- No-till was associated with higher soil organic matter and soil N uptake, supporting improved legume N status.
- No-till alone was insufficient to prevent N limitation in hairy vetch under unfertilized conditions.

Equation (2), and the %N_c was estimated using Equation (3):

$$\text{NNI} = \frac{\%N}{\%N_c} \quad (2)$$

$$\begin{aligned} \%N_c &= 4.8 \times \text{DM}^{-0.32} \text{ (for DM > 1 Mg ha}^{-1}\text{)} \\ \%N_c &= 4.8 \text{ (for DM} \leq \text{1 Mg ha}^{-1}\text{)} \end{aligned} \quad (3)$$

Previously collected SOM data from 2013, corresponding to 7.5-cm depth soil samples, were used to explore relationships with plant N sources, status, and fixation.

2.3 | Statistical analysis

To assess the effects of tillage and N fertilization on plant traits (NNI, Nd_{fa}, kg N fixed, kg N from soil, total plant N, and DM) and SOM, data from both years were combined and analyzed using linear mixed-effects models in R (*lme4* package, version 4.3.0; Bates et al., 2015). Tillage and N fertilization were treated as fixed effects, while block and year were included as random effects. Where main effects or interactions were significant (α = 0.05), pairwise comparisons were conducted using estimated marginal means (*emmeans* package; Lenth, 2025) with Tukey's Honestly Significant Difference. Variables were log or square root transformed as needed to meet model assumptions.

To examine multivariate relationships between SOM and plant N variables, a redundancy analysis (RDA) was conducted using standardized values of NNI, Nd_{fa}, kg N fixed, kg N from soil, and SOM. The model was constrained by tillage and fertilization treatments, with block and year included as covariates. The RDA was performed in Python using the *scikit-learn* library (version 3.10.12; Pedregosa et al. [2011]).

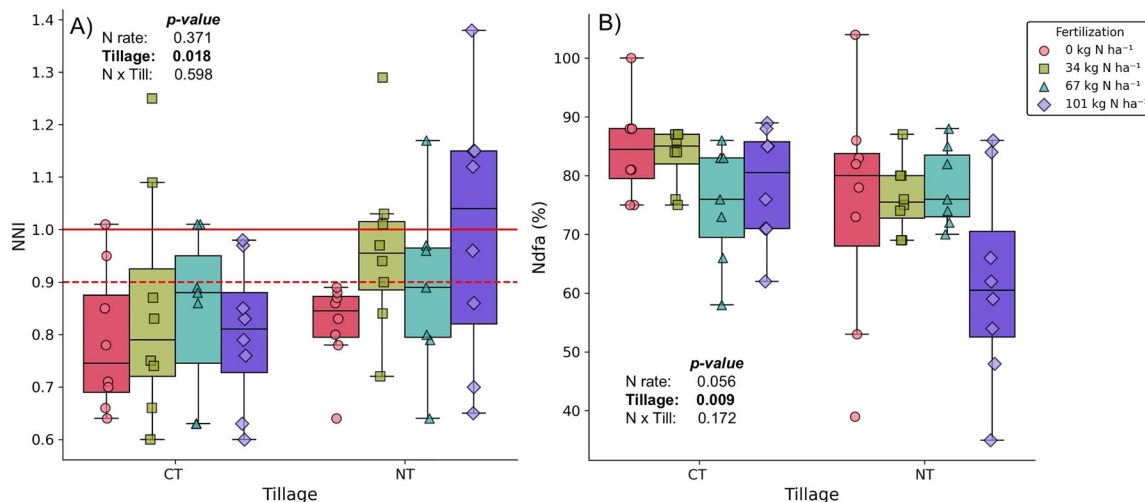


FIGURE 1 Scattered boxplots showing the effects of fertilization and tillage on (A) nitrogen nutrition index (NNI) and (B) proportion of nitrogen derived from the atmosphere (Ndfa). Points represent individual observations; boxes indicate the interquartile range, with median shown as horizontal lines. In panel A, the solid red line indicates NNI = 1, representing optimal nitrogen status, while the dashed red line marks NNI = 0.9, below which nitrogen limitation become more pronounced.

3 | RESULTS AND DISCUSSION

This study evaluates how legacy effects of long-term tillage and N fertilization influence N status, BNF, and N acquisition strategies in vetch within a continuous cotton system. Across N treatments, vetch grown under CT exhibited a lower NNI compared to NT plots (mean: 0.82 vs. 0.92), reflecting a significant main effect of tillage (Figure 1A). Although the N rate $\times$ tillage interaction was not significant, generally higher NNI with increasing N fertilization rates was notable across both tillage treatments, except at 67 kg N ha⁻¹ (Figure 1A). Differences in NNI between CT and NT were minimal under unfertilized conditions (mean: 0.79 vs. 0.82), with both values below 0.85, indicating a marked N limitation for vetch growth regardless of tillage treatment. These findings support the hypothesis that NT systems enhance soil N supply, likely due to the higher SOM accumulation (Figure S1) and thus greater availability of soil-derived N (Table 1). However, the comparable NNI levels and overlapping distributions under 0 N also suggest that NT alone may not be sufficient to fully sustain legume N status in the absence of fertilization, underscoring the importance of adequate N management across the entire crop rotation sequence to prevent soil fertility depletion and avoid N limitation not only for the cash crop but also during the CC phase.

The %Ndfa was statistically higher in vetch from CT plots compared to NT plots (mean: 80.5 vs. 72.5%), and although the N rate $\times$ tillage interaction was not significant, a general trend of decreasing %Ndfa with increasing N fertilization level was observed, except at 67 kg N ha⁻¹ (Figure 1B).

This pattern suggests a compensatory increase in %Ndfa in CT plots, where soil N availability is lower due to SOM depletion from continued tillage and reduced mineralization potential (Peoples et al., 1995; Reiter et al., 2002). Conversely, NT plots showed significantly higher total N uptake (Table 1), which, together with greater SOM content (Figure S1), indicates enhanced soil N supply through increased mineralization potential and nutrient retention, leading to greater availability of soil-derived N to the CC (Table 1). This is consistent with known suppression of %Ndfa under elevated soil nitrate (Sun et al., 2019; Unkovich, 2012), further emphasizing the role of soil N status in regulating N fixation. This trade-off between soil N uptake and %Ndfa has been previously reported across legume systems (Doughton et al., 1993; Matus et al., 1997) and represents a key mechanism by which legumes maintain N status and avoid N limitations across diverse environments.

While vetch aboveground biomass production remained unaffected by tillage or fertilization (Figure S2), tillage strongly influenced soil N supply. Total N uptake was significantly higher under NT compared to CT, primarily due to greater soil-derived N availability, while the amount of atmospheric N fixed did not differ between systems (Table 1; Figure S3). Conversely, long-term application of different N rates had a less pronounced effect: total N uptake by vetch remained unchanged across fertilization rates. However, the relative contributions of soil and atmospheric sources shifted with increasing N input. Higher N application to the preceding cotton crop significantly increased the amount of N in vetch derived from soil, while the amount of atmospheric N fixed

TABLE 1 Effect of nitrogen fertilization rate and tillage on nitrogen sources in hairy vetch, expressed as the amount of nitrogen (kg N) per Mg of dry matter in plant biomass.

Treatments	Sources of nitrogen in legumes		
	kg N fix (kg N Mg ⁻¹)	kg N soil (kg N Mg ⁻¹)	kg N total (kg N Mg ⁻¹)
N rate			
0	26.2 (±8.7)	6.6 (±5.3)b	32.7 (±6.7)
34	28.4 (±7.1)	7.0 (±2.4)ab	35.4 (±7.4)
67	24.2 (±5.0)	8.0 (±3.6)ab	32.2 (±4.5)
101	23.6 (±4.5)	11.1 (±7.7)a	34.7 (±6.7)
Tillage			
CT	25.4 (±6.1)	6.3 (±3.3)b	31.7 (±5.7)b
NT	25.9 (±7.3)	10.0 (±6.4)a	35.9 (±6.5)a

Note: Lowercase letters indicate significant treatment effects (p -value ≤ 0.05). Values represent means averaged across the 2 years of the experiment.

Abbreviations: CT, conventional tillage; NT, no-till.

per unit biomass marginally declined ($p > 0.05$) with increasing N rate, indicating a compensatory role by soil N supply (Table 1).

The RDA provided further insight into treatments effects, with the first two axes explaining 83.2% of the variance in N-related variables (Figure 2). RDA1 primarily captured a gradient in N source and status: higher NNI, soil-derived N, and SOM were associated with more negative scores, while higher %Ndfa aligned with more positive scores (Table S2). This reflects a tradeoff between reliance on soil-derived N and atmospheric N fixation, with treatments positioned along this axis according to their dominant N source. NT treatments clustered on the negative side of RDA1—NT-101N, NT-67N, NT-34N, and NT-0N—following a gradient of increasing soil-derived N and N status with greater fertilization. CT treatments, in contrast, were distributed along the positive side—CT-0N, CT-34N, NT-67N, and CT-101 N—reflecting lower soil N supply and a stronger dependence on BNF, particularly under low N inputs. This separation is consistent with the long-term benefits of NT in enhancing SOM and soil N availability (Zakeri et al., 2012) and with the inhibitory effects of elevated soil nitrate on BNF (Peoples et al., 1995; Sun et al., 2019).

RDA2 captured variation related to BNF efficiency and SOM, where more positive scores correspond to higher kg N fixed per Mg DM and greater SOM. Treatments such as NT-34N, NT-0N, NT-101N, and NT-67N were associated with higher RDA2 values, reflecting both enhanced SOM and, for NT-0N and NT-34N, higher N fixation efficiency under low N input. In contrast, CT treatments generally showed lower RDA2 scores, indicating reduced SOM under CT, though CT-0N and CT-34N still aligned with greater BNF efficiency under low fertilization.

Overall, the RDA revealed clear management-driven patterns in legume N acquisition strategies. NT systems aligned

with greater SOM, higher soil-derived N uptake, and improved legume N status, whereas CT was characterized by lower SOM and a greater compensatory reliance on atmospheric N fixation. Although fertilization effects were secondary to tillage, lower N inputs consistently promoted higher BNF efficiency. Together, these results demonstrate how long-term tillage and fertilization treatments regulate the balance between soil- and atmosphere-derived N, shaping legume N status and soil N sustainability in continuous cotton systems.

4 | CONCLUSIONS

Our findings demonstrate that long-term management practices shape N acquisition strategies in legume CCs within continuous cotton systems. The combined effects of tillage and N fertilization influenced the balance between soil- and atmosphere-derived N in vetch. NT promoted greater SOM accumulation and, hence, overall higher soil-derived N uptake, while CT was associated with lower SOM and increased reliance on BNF, particularly under low fertilization. Although total N input from vetch was not significantly affected by fertilization, the relative contribution of atmospheric N increased under reduced N inputs, indicating a compensatory shift in fixation efficiency. This pattern, consistent in both tillage systems and supported by the RDA results, suggests that legume CCs can partially offset reduced fertilizer inputs through enhanced BNF.

These results highlight the importance of long-term soil health practices, particularly NT, in improving soil fertility and supporting legume N status in nutrient-limited systems. While legumes can rely on BNF, their N status remains closely tied to soil mineral N availability. CCs

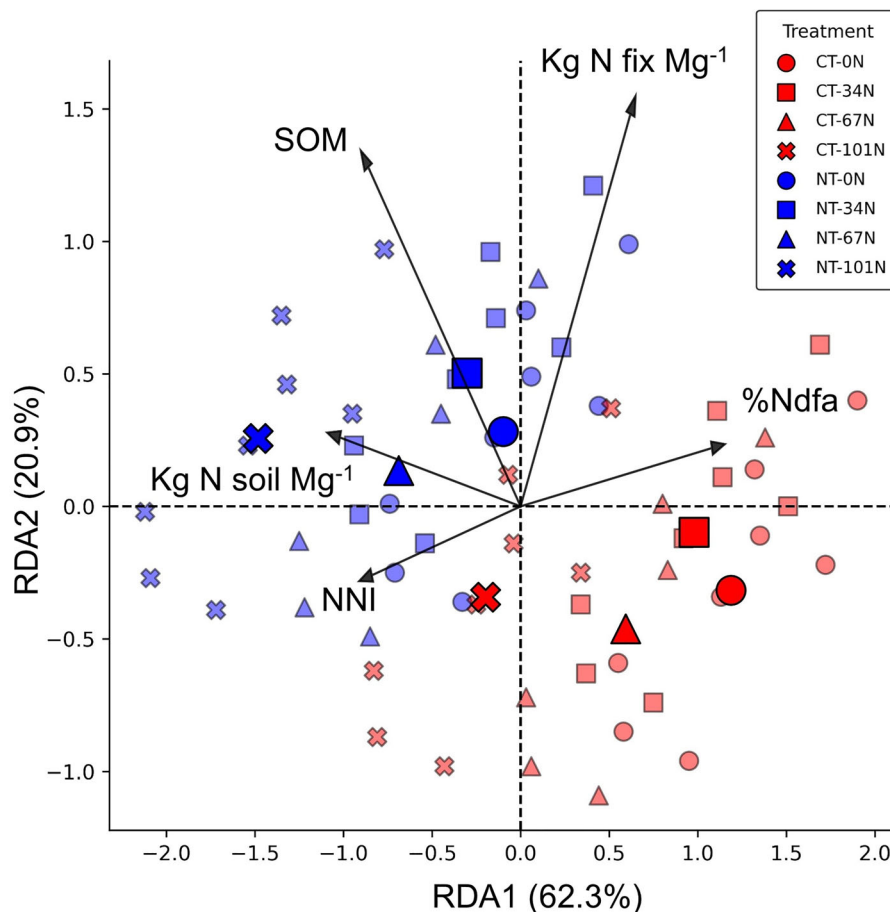


FIGURE 2 Redundancy analysis (RDA) biplot showing individual samples and treatments centroids based on nitrogen-related variables. Transparent markers represent individual observations, while solid, enlarged markers indicate group centroids for each tillage × fertilization treatment combination. Black arrows represent the response variables (nitrogen nutrition index [NNI], nitrogen derived from the atmosphere [Ndfa], kg N fix, kg N soil, and soil organic matter [SOM]), with direction and length indicating their contribution to the constrained ordination axes. CT, conventional tillage; NT, no-till.

exhibited flexible N acquisition strategies, adjusting their reliance on BNF and soil-derived N across fertilization levels. However, in unfertilized systems, legume N status markedly declined ($NNI < 0.85$), highlighting the need for adequate N fertilization throughout the crop rotation even under NT. Overall, these findings support the integration of NT with fertilization rates adjusted to the crop rotation as part of sustainable nutrient management to sustain legume performance, soil function, and long-term agricultural resilience.

AUTHOR CONTRIBUTIONS

Facundo Lussich: Conceptualization; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **Jashanjeet Kaur Dhaliwal:** Supervision; writing—review and editing. **Sindhu Jagadamma:** Funding acquisition; supervision; writing—review and editing. **Debasish Saha:** Conceptualization; funding acquisition; inves-

tigation; methodology; project administration; resources; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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