Soil C:N impacts on soil biological health and consequences on weed control in soybean and corn systems

Corresponding author:
Jenny Kao-Kniffin; Email: jtk57@cornell.edu

Maria A. Gannett[1] , Aleah L. Butler-Jones[1] , Antonio DiTommaso[2] ,
Jed P. Sparks[3] and Jenny Kao-Kniffin[4]

[1]Graduate Student, Cornell University, Ithaca, NY, USA; [2]Professor, School of Integrative Plant Science, Soil and Crop Science Section, Cornell University, Ithaca, NY, USA; [3]Professor, Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA and [4]Associate Professor, School of Integrative Plant Science, Horticulture Section, Cornell University, Ithaca, NY, USA

Abstract

Nitrogen availability has an important influence on agricultural weed growth, because many weeds in annual cropping systems are more competitive in high-nitrogen soils. A potential method to control nitrogen availability is through soil carbon amendments, which stimulate soil microbial growth and immobilize nitrogen. Additionally, carbon amendments may alter soil microbial community composition, increase soil biological functioning, and improve soil health. In a 2-yr field experiment in corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.], we implemented five amendment treatments to test their ability to alter weed and crop growth through soil nitrogen availability and soil biological functioning. The treatments included: an untreated control, an unamended weed-free control, rye hay adding 3,560 kg C ha^{-1} and 3,350 kg C ha^{-1} in 2020 and 2021, respectively, sawdust adding 5,030 kg C ha^{-1} and 4,350 kg C ha^{-1} in 2020 and 2021, respectively, and a rye hay and sawdust combined treatment adding 8,590 kg C ha^{-1} and 7,700 kg C ha^{-1} in 2020 and 2021, respectively. Each treatment was replicated five times in corn and six times in soybean. Each season, we explored correlations between crop and weed biomass and weed community composition and nitrogen immobilization measured through soil respiration and nitrogen availability. We also explored changes to the soil microbial community composition and soil health as a secondary result of the carbon amendment treatments. Nitrogen availability was lowest in plots treated with the highest C:N amendment. Increasing carbon improved soil health metrics, but the microbial community composition was most affected by the rye hay treatment. Amendments with high C:N reduced weed growth in both soybean and corn plots but only selected for specific weed communities in soybean, leading to improved soybean competitiveness against weeds. In corn, crop growth and weed community composition remained consistent across amendment treatments. Targeted nitrogen immobilization may improve leguminous crop competition in some weed communities as part of an integrated weed management program.

Introduction

An important aspect of weed control is accurate fertilization. In annual cropping systems, the most abundant weeds are nitrophilous, meaning that they grow well in high-nitrogen soils (Costea et al. 2004; Little et al. 2021; Moreau et al. 2014). Some of these species can outcompete crops for nitrogen and have higher nitrogen use efficiencies, which can lead to lower crop yields in fertilized soil relative to unfertilized soil at high weed densities (Di Tomaso 1995; Little et al. 2021). Once a weed accumulates more nutrients than a crop, it may more strongly compete for additional resources, such as light (Di Tomaso 1995). Weeds can be especially competitive over crops with inorganic fertilizer use (Saberali and Mohammadi 2015).

Previous research has focused on varying the rates, forms, timing, and location of fertilizer applications to reduce nutrient uptake by weed species and enhance the competitive ability of crops over weeds. Studies that vary fertilization rates have produced mixed results depending on the nitrogen responsiveness of the crop and weed species (Blackshaw and Brandt 2008). In some cases, reduced fertilization increased crop yield by reducing competition from weeds (Blackshaw et al. 2003; Davis and Liebman 2001; Wortman et al. 2011). In other cases, there was no effect of nutrient availability on crop yield (Barker et al. 2006; Wortman et al. 2011). And in still others, crop yield increased with increased fertilization rates, despite increased weed growth (Anderson et al. 1998; Juroszek et al. 2004). Little et al. (2021) developed a framework to help predict the effects of fertilization on weed competition and crop yield. According to this framework, key sources of variation in the effects of fertilization on crops and weeds include relative

responsiveness to added nutrient, relative competitiveness, and relative shading ability under high fertility.

Another strategy to manage the growth of nitrophilous weeds is through nitrogen immobilization. Nitrogen immobilization is a microbially mediated process (Schimel and Bennett 2004). Carbon amendments added to the soil increase the growth of the soil microbial community (Zak et al. 1994). As the microbes grow, they also take up available soil nitrogen to maintain their stoichiometric ratio of carbon to nitrogen. This process leaves less nitrogen available to plant roots and may reduce weed growth.

Nitrogen immobilization may contribute to the efficacy of several existing agricultural weed management strategies that utilize high-carbon substrates. Many perennial cropping systems, such as strawberries [*Fragaria* ×*ananassa* (Weston) Duchesne ex Rozier ssp. *ananassa*] (Pritts and Handley 1998), raspberries (*Rubus idaeus* L.) (Trinka and Pritts 1992), blueberries (*Vaccinium corymbosum* L.), and apples [*Malus domestica* (Suckow) Borkh.] (Merwin et al. 1995), rely on high-carbon mulches to help manage weeds. Terminated cover crops can also immobilize nitrogen, and nitrogen immobilization may contribute to their weed-suppressive ability (Pittman et al. 2020; Wells et al. 2013; Williams et al. 2018). Mechanisms other than nitrogen immobilization, including physical impedance (Creamer et al. 1996; Teasdale and Mohler 2000), light quantity and quality (Teasdale 1993), changing microclimatic conditions (Teasdale et al. 2007), and allelopathy (Scavo and Mauromicale 2021), certainly contribute to weed suppression by these mulches. Nitrogen immobilization, however, likely plays an important role in all these strategies, and it is worth exploring in isolation. Managing plant growth through nitrogen immobilization has been studied as a tool to restore native plant growth in natural ecosystems invaded by nonnative plants but has not yet been assessed in cropping systems. A more in-depth understanding of how nitrogen immobilization affects crop and weed growth may provide future weed management opportunities in cropping systems.

Adding carbon to soil to induce nitrogen immobilization may also alter the composition of the soil microbial community, soil biological functioning, and soil health (Lal 2014, 2016). Good soil biological functioning is associated with diverse benefits such as improved aggregate stability, leading to increased water-holding capacity, porosity, and water infiltration; increased nutrient cycling; improved CEC; and increased crop yields (Luo et al. 2018; Thangarajan et al. 2013). The specific microbial composition can also play an important role in modulating plant growth (Chaparro et al. 2012). Many bacterial and fungal endophytes, saprophytes, hyperparasites, and arbuscular mycorrhizal fungi can induce plant defense mechanisms, helping protect plants from disease (Raaijmakers et al. 2009). Some microbes are microbial antagonists or microbial parasites, which help protect plants from pathogenic microbes. Many *Trichoderma*, *Gliocladium*, and nonpathogenic *Fusarium* (all Ascomycota) are antagonistic fungi (Harman et al. 2004; Raaijmakers et al. 2009). Many of the *Pseudomonas* (Proteobacteria), *Burkholderia* (Proteobacteria), *Bacillus* (Firmicutes), and *Actinobacteria* are antagonistic bacteria (Barka et al. 2015; Raaijmakers et al. 2009). *Bacillus* can also improve macronutrient cycling by solubilizing phosphorous-containing potassium compounds (Song et al. 2019; Wang et al. 2017). It is likely that stimulating nitrogen immobilization with high carbon amendments will alter soil microbial community composition and soil health functioning, but it is unclear how those changes will affect weed management.

There were two main objectives of this research. The first objective was to isolate and assess the efficacy of nitrogen immobilization as a tool for managing weeds in different field cropping systems. We hypothesized that amendments with higher carbon:nitrogen ratios (C:N) added to the soil would increase nitrogen immobilization and reduce weed growth to a greater extent than lower C:N. This suppression would be greater for nitrophilous weeds, thus altering weed community composition. We predicted that soybean [*Glycine max* (L.) Merr.] would be more competitive in nitrogen-immobilized soils, as soybeans can fix atmospheric nitrogen and are less reliant on soil nitrogen availability. We predicted that corn (*Zea mays* L.) growth would decrease as nitrogen immobilization increased, because modern corn varieties are highly responsive to nitrogen (Liu et al. 2022) and have higher recommended nitrogen rates than many other crops (Mylavarapu et al. 2021). The second objective of this study was to explore how high rates of carbon addition would change soil microbial community composition and soil health functioning. We hypothesized that higher carbon inputs would stimulate greater microbial growth and activity and alter microbial community composition. We predicted that these compositional changes would be correlated with improved soil health and function and may be a multifunctional benefit associated with nitrogen immobilization.

Material and Methods

Field Set-Up

Field trials were initiated in the spring of 2020 at the Homer C. Thompson Vegetable Research Farm in Freeville, NY, USA (42.516965°N, 76.334245°W). The soil is classified as Howard gravelly loam (loamy-skeletal, mixed, active, mesic Glossic Hapludalfs) (https://websoilsurvey.nrcs.usda.gov/app/). Baseline soil samples were submitted to the Cornell Nutrient Analysis Laboratory at the beginning of the study, and nutrient levels were within recommended ranges for corn and soybean (Supplementary Table S1). The field used for both corn and soybean plots was previously planted to spinach (*Spinacia oleracea* L.) in 2019 and was seeded with winter rye (*Secale cereale* L.) in autumn of 2019 and 2020. In early May 2020 and 2021, rye was chopped at the transition phase (about 15 to 20 cm height) and removed from the field, and then the field was plowed to prepare for treatments and planting. This rye hay, in addition to rye planted and cut at the same time in a neighboring field, was used for the rye hay amendment. Sawdust was collected from a local sawmill (42.2658°N, 76.6461°W) and consisted of about 90% white pine (*Pinus strobus* L.) and hemlock [*Tsuga canadensis* (L.) Carrière]. The amendments were spread and incorporated into the soil within 3 wk of rye harvest, and therefore the rye hay was still green and wet when used as an amendment (77% and 57% moisture in 2020 and 2021, respectively). Soybean and corn were planted separately. For each crop, plots were arranged in a complete randomized block design. Within each block, there were five treatments: sawdust amended, rye hay amended, rye hay and sawdust combined, nontreated control, and unamended weed-free control. Soil amendment rates and carbon and nitrogen characteristics are listed in Table 1. Amendments were spread starting mid-May, rototilled to about 15-cm depth, and then soybean and corn were planted on May 27 in 2020 and May 24 in 2021 in rows oriented north to south. Rainfall was below average in 2020, so supplemental irrigation was provided as needed. In 2021,

Table 1. Carbon (C) and nitrogen (N) content of amendments added to the soil in 2020 and 2021 (Homer C. Thompson Vegetable Research Farm in Freeville, NY, USA)

	Fresh biomass	Dry biomass	C	N	C:N	Application rate[a]	
	g	g	%	%		kg C ha^{-1}	kg N ha^{-1}
2020							
Rye hay	40,850	9,390	45.5	2.4	19	3,560	190
Sawdust	38,610	13,200	45.7	0.2	288	5,030	20
Sawdust + rye hay	79,460	22,590	45.6	1.1	41	8,590	209
2021							
Rye hay	41,940	18,000	42.8	2.1	21	3,350	160
Sawdust	42,430	11,220	39.6	0.2	221	4,350	20
Sawdust + rye hay	84,370	29,250	41.6	1.4	30	7,700	260

[a]To convert to g m^{-2}, multiply by 0.1.

rainfall was above average, so no supplemental irrigation was used (Supplementary Figure S1).

Soybeans were planted at 76-cm between-row and 4-cm within-row spacing. The soybean planting was divided into six blocks. Each block contained all five amendment treatments. Soybean plots were 6-m wide and 4-m long, contained eight crop rows, and included a split-plot treatment in which half of each plot (four crop rows) was inoculated with rhizobia and half remained uninoculated. The soybean variety was 01072245 Roundup Ready 2 Xtend®, Roundup Ready 2 Yield® Technology (Asgrow® AG20X9, Bayer, Whippany, NJ), which was not preinoculated. Soybeans planted in inoculated plots were inoculated with rhizobia at a rate of 9.4 g kg^{-1} seed (N-Dure™ Soybean, Verdesian, Cary, NC) about 10 min before planting. However, at harvest in 2020, we dug three soybean plants from each plot, washed the roots, and counted root nodulation. There was no difference in nodulation rate by inoculation treatment (df = 1, F-value = 0.004, P-value = 0.95). This finding indicates that there was an endemic population of *Bradyrhizobium japonicum* in the soil that successfully colonized the non-inoculated soybeans, reducing the effect of the inoculation treatment. Therefore, the split-plot inoculation treatment was sometimes removed from analysis when it was not significant. Removing the split-plot treatment effectively increased the number of soybean crop replicates to 12 for several of the analyses.

There were five blocks of silage corn, and each block contained all five amendment treatments for a total of 25 plots. Each plot was 3-m wide and 4-m long to accommodate four crop rows. The corn was a hybrid blend of 95% 'FS 46R64VT2P' and 5% 'VNS RR' (VTDouble PRO Rib Complete, Monsanto, St Louis, MO). Corn was planted at 76-cm between-row and 20-cm within-row spacing. At planting, 224.2 kg ha^{-1} of nitrogen from 10-20-20 banded fertilizer (plant blended, Phelps Supply, Phelps, NY) was applied at a depth of 5 cm, which is on the high end of regional application rates. Although the goal was to immobilize nitrogen, corn is known to require substantial amounts of nitrogen fertilization. We hoped that band fertilizing could provide adequate nutrients to corn plants, while still allowing for nitrogen immobilization to suppress weeds between rows.

Weed Communities

Seeds from four weed species were scattered in each plot in early June 2020 (not in 2021): Powell amaranth (*Amaranthus powellii* S. Watson), velvetleaf (*Abutilon theophrasti* Medik.), white clover (*Trifolium repens* L.), and ivyleaf morningglory (*Ipomoea hederacea* Jacq.). Seeds had all been collected from local farms and were cold stratified for at least 1 yr at 4 C. This is somewhat late

for seedbank spiking; however, we wanted to wait until after plots had been rototilled so as not to bury the seeds too deep within the soil profile. Preweighed seeds from all four species (Supplementary Table S2) were combined into a screw-top bottle (HDPE Plastic Wide Mouth, Thermo Scientific Nalgene, Rochester, NY), mixed with 120 ml of paver sand (Multi-purpose Patio/Paver Sand, Pavestone, Atlanta, GA) to consistently distribute the different-sized seeds within the bottle, and then distributed evenly across each plot. All plots were raked to improve seed–soil contact. *Amaranthus powellii* and *A. theophrasti* were expected to have reduced growth in carbon-amended soils, as these species are known to be responsive to nitrogen (Mohler et al. 2021). *Trifolium repens* was not expected to show a strong response, as it is a legume and may be less responsive to nitrogen. We were unsure of the response of *I. hederacea*, which has a high Ellenberg N index, but which showed a reduced response to low nitrogen soils in previous research in our lab. This seedbank spike, however, was largely unsuccessful. All species made up 2.6% or less of the total weed biomass each year, except *A. powellii*, which had resident populations (Supplementary Table S2).

Weed surveys were conducted in early August of each year of the experiment. A 0.25-m^2 quadrat was placed 1 m into the third crop row of each plot from the southeast corner. The quadrats were 0.33-m wide and 0.75-m long and straddled the crop row, with 0.375 m extending into the interrow space. In each quadrat, all weeds with at least one true leaf were identified to species and clipped at the soil surface, dried at 60 C to a constant weight, and then weighed. Only weeds rooted within the quadrat were counted.

Field Measurements

Soil nitrogen availability was measured using a method adapted from the Kellogg Biological Station Long-Term Ecological Research program (http://lter.kbs.msu.edu/protocols/105). Anion and cation resin exchange membranes (Membranes International, Ringwood, NJ) were cut into strips 10-cm long and 2.5-cm wide. To charge the exchange strips, they were soaked in a 0.5 M HCl bath for 1 h, with stirring every 10 min. Then, strips were rinsed with ultrapure water and soaked in a 0.5 M NaHCO$_3$ bath for 5 h, which was changed every hour. Strips were rinsed with ultrapure water a final time and stored at 4 C in sealed plastic bags to prevent drying. Anion and cation strips were charged and stored separately.

Strips were deployed in the field for periods of 3 wk. In 2020, two measurements were taken in late June and early August. In 2021, four measurements were taken in late June, mid-July, early August, and late August. To best measure root available nitrogen, strips were buried vertically 9.5-cm deep and approximately 15 cm

Table 2. P-values, degrees of freedom (df), and *F*-values of crop and weed aboveground biomass from a type II ANOVA of a linear model for each independent variable included in the model

Independent variable	df	*F*-value	P-value	df	F-value	P-value
		Soybean			Corn	
Amendment	4	6.9741	<0.0001	4	2.2116	0.08691
Inoculation	1	2.3586	0.12766	N/A	N/A	N/A
Weed biomass	1	1.2323	0.26955	1	0.8832	0.35341
Year	1	6.8523	0.01019	1	3.6753	0.06316
Amendment × inoculation	4	0.6193	0.64978	N/A	N/A	N/A
Amendment × weed biomass	4	1.9027	0.1156	4	0.6902	0.6034
		Weeds of soybean plots			Weeds of corn plots	
Amendment	4	146.61097	<0.0001	4	68.27369	<0.0001
Inoculation	1	1.09508	0.2978	N/A	N/A	N/A
Year	1	0.06571	0.7982	1	0.40776	0.5267
Amendment × inoculation	4	0.48764	0.7448	N/A	N/A	N/A
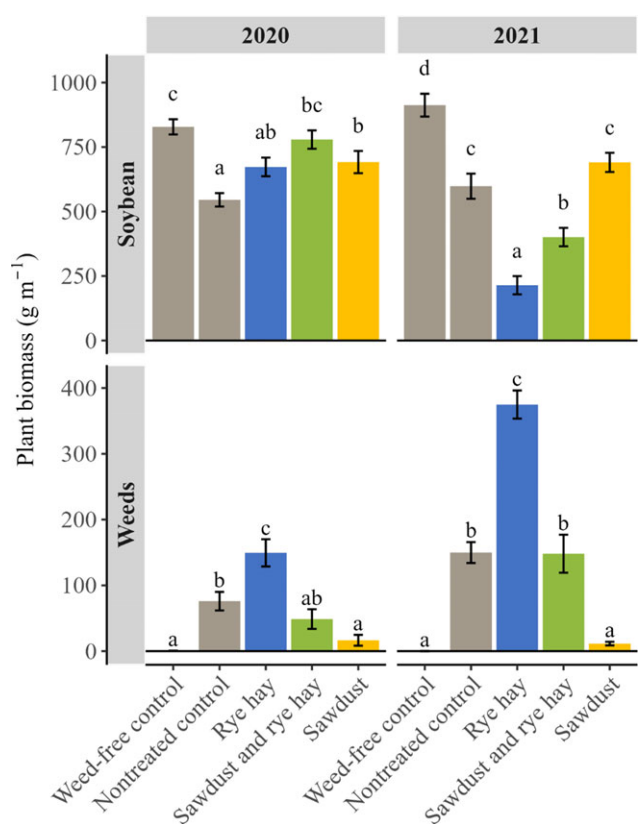
Figure 1. Soybean crop yield and total weed biomass in soils with different amendments. Bars labeled with different letters indicate significantly different means (P < 0.05) by treatment.

on either side of the crops in the between-row space. Care was taken to reduce soil disturbance and to ensure good strip–soil contact. After collection from the field, strips were rinsed with ultrapure water to remove visible soil particles and stored at 4 C until extraction. Any strips that had been partially eaten by rodents in the field were measured to determine the area of strip missing.

Soil respiration was measured using a soil gas flux survey chamber and CO_2 analyzer (Smart Chamber and LI-870 CO_2/H_2O Analyzer, Li-Cor, Lincoln, NE). Soil collars were made from SDR35 PVC pipe, 20 cm in diameter and 10-cm tall. Collars were installed in the field in late July 2020 and early June 2021, leaving about 1 to 3 cm of the collar above the soil surface. The average offset height of each collar was integrated into the measurement of respiration per soil volume over 120 s. Respiration was measured twice in 2020 (late July and August) and four times in 2021 (mid-June, July, August, and early September). Measurements were taken on consecutive mornings for corn and soybean, at least 5 d after collar installation and at least 24 h after removing any weeds from the collar.

Crop Analyses

Corn and soybean samples were harvested in late September to early October 2020 and late September 2021. Samples were collected from the third crop row of each plot relative to the southeast corner, the row next to which weeds had been collected. Within a 2-m section in the middle of the row, all aboveground crop biomass was harvested, counted, and then dried at 60 C to a constant weight. Soybean pods were shelled by hand in 2020 and with a thresher in 2021 (LD 350, Wintersteiger, Innkreis, Austria) to measure seed yield. The entire corn stalk was weighed together. In 2020, the roots of three soybean plants within each harvested row were removed from the soil, carefully washed, and photographed. Root nodules were counted from the photographs.

Lab Analyses

Before harvest, two soil samples were collected and homogenized from each corn and soybean plot. Samples were collected from the between-row space of the two middle rows, using a 2-cm-wide and 13-cm-long soil core sampler (AMS Inc., Americana Falls, ID). One sample from each plot was collected and discarded before keeping samples to "rinse" the core. Samples were stored on ice in the field and then transferred and stored at 3 C (Setpoint temperature control model SP-322, Goldline, North Kingstown, RI) until processed.

Soil was sieved through a 2-mm sieve and further divided for future analyses. One aliquot was frozen at −20 C to be used for DNA extraction, while another aliquot was set out to air dry. Sieves were rinsed with water, wiped with ethanol, and dried between samples. Gravimetric soil moisture was calculated by weighing a 10-g subsample of fresh soil into a tin cap and drying at 105 C in an oven (Isotemp Oven, Fisher Scientific, Suwanee, GA) for 24 h before reweighing.

To measure pH, 3.0 ± 0.1 g of air-dried soil was mixed with 6 ml of deionized water in a 15 ml falcon tube and shaken on a reciprocal shaker (E6000 Medium-Duty, Eberbach, Ann Arbor, MI) at 180 rpm for 60 min. Tubes were removed from the shaker and allowed to sit for at least 10 min before pH measurements were taken with a pH meter (Orion Star A215, Thermo Scientific, Waltham, MA).

Table 3. Significant indicator species identified in plots with different amendment treatments

Year	Treatment group	Weed species	Specificity[a]	Sensitivity[b]	Stat	P-value
		Soybean				
2020	Rye hay	*Amaranthus* spp.	0.82	1.00	0.91	0.005
	Nontreated control + rye hay	*Capsella bursa-pastoris*	0.97	0.79	0.88	0.005
	Nontreated control + rye hay + sawdust and rye hay	*Portulaca oleracea*	1.00	0.92	0.96	0.005
2021	Rye hay	*Amaranthus* spp.	0.80	0.92	0.86	0.005
		Chenopodium album	0.83	0.75	0.79	0.005
	Nontreated control + rye hay	*Capsella bursa-pastoris*	0.96	0.96	0.96	0.005
	Rye hay + sawdust and rye hay	*Stellaria media*	1.00	0.63	0.79	0.005
		Dandelion (*Taraxacum officinale* F.H. Wigg.)	0.95	0.54	0.72	0.025
	Nontreated control + rye hay + sawdust and rye hay	*Panicum capillare*	1.00	0.86	0.93	0.010
		Portulaca oleracea	0.98	0.81	0.89	0.005
		Solanum physalifolium	1.00	0.42	0.65	0.025
		Corn				
2020	Nontreated control + Rye hay	*Capsella bursa-pastoris*	0.91	0.90	0.90	0.020
	Sawdust and rye hay + sawdust	*Digitaria sanguinalis*	0.94	1.00	0.97	0.005
	Nontreated control + rye hay + sawdust and rye hay	*Portulaca oleracea*	0.98	0.93	0.96	0.030

[a]High specificity value indicates that species occurs in plots belonging to this treatment only.
[b]High sensitivity value indicates that species appears in all plots belonging to this treatment.

Soil carbon and nitrogen were measured using air-dried and finely ground soil. The grinding apparatus was rinsed with ethanol between samples to prevent cross-contamination. About 30 mg of powdered soil was weighed into tin capsules and submitted to the Cornell Stable Isotope Lab for percent carbon (C), nitrogen (N), ^{13}C, and ^{15}N analysis on an isotope ratio mass spectrometer (Delta V, Thermo Scientific) connected to an elemental analyzer (NC2500, Thermo Scientific).

Both anion and cation resin strips were mixed with 70 ml of 1 M KCl in an acid-washed sample cup. Cups were shaken for 24 and 22 h in 2020 and 2021, respectively, at 180 rpm on a reciprocal shaker (MaxQ 4000, Thermo Scientific) then decanted into 15-ml falcon tubes. Collected extract from all samples was stored frozen at −20 C until analysis. Nitrate and ammonium were quantified using the colorimetric method described by Hood-Nowotny et al. (2010). Samples were analyzed on a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT) and compared with a standard curve to calculate concentration.

In 2021, subsets of soil collected from the non-inoculated soybean and corn plots were submitted to the Cornell Soil Health Lab for a soil health assessment. Soil health indicator tests included: aggregate stability, organic matter, autoclaved-citrate extractable (ACE) soil protein index, active carbon, and macro- and micronutrients. Depth to compaction was also measured by recording the depth at which 21.1 kg cm^{-2} of penetration resistance was reached using a penetrometer (AgraTronix Soil Compaction Tester, Streetsboro, OH) (Duiker 2002).

Microbial Amplification

Bacterial and fungal community composition were assessed using high-throughput amplicon sequencing. DNA was extracted from approximately 200 mg of soil using the Qiagen DNeasy soil DNA extraction kit (Beverly, MA) following the manufacturer's instructions. The V3-V4 region of the 16S rRNA gene (16S) was targeted using 341F and 805R primers for bacterial identification (Herlemann et al. 2011). The ITS2 region of the fungal internal transcribed spacer gene (ITS) region was targeted using the ITS1F and 58A2R primers for fungal identification (Gardes and Bruns 1993; Martin and Rygiewicz 2005). Amplified samples were cleaned, indexed, and pooled following the procedure detailed by Garcia et al. (2022). Pooled samples were sequenced on the Illumina MiSeq at the Cornell Genomics Facility (Ithaca, NY) using a 500-cycle MiSeq Reagent Kit v. 2 for the ITS pool and a 600-cycle MiSeq Reagent Kit v. 3 for the 16S pool. The raw sequencing data for both 16S and ITS regions of the soil microbial community were deposited into the National Center for Biotechnology Information Sequence Read Archive under BioProject ID: PRJNA1005696.

Sequence merging and filtering were performed in Qiime2. Reads were demultiplexed, and paired ends were merged and trimmed of primers using q2-demux. Sequences were then denoised into amplicon sequence variants (ASVs) using DADA2 (Callahan et al. 2016). The ASVs were clustered using de novo q2-vsearch, with 97% shared identity as the cutoff, to create a table of assigned operational taxonomic units (OTUs). The OTU table was used to create a feature table using q2-feature-table. Bacterial and fungal classifiers were trained with a Naïve Bayes classifier trained on the 99% Silva 138 database for bacteria (Bokulich et al. 2018; Robeson et al. 2021) and the UNITE 13.8 database for fungi (Abarenkov et al. 2010), and then taxonomy was assigned to sequences in the taxonomy table using classify-sklearn.

Statistical Analyses

All statistical analyses were performed in R v. 4.1.2 (R Core Team 2022). Corn and soybean yields were analyzed separately using linear mixed-effects models (function *lmer*, in package LME4 v. 1.1-31) with soil amendment treatment, year, weed biomass, and the interaction of weed biomass and amendment as fixed effects. The model for soybean yield also included an inoculation treatment
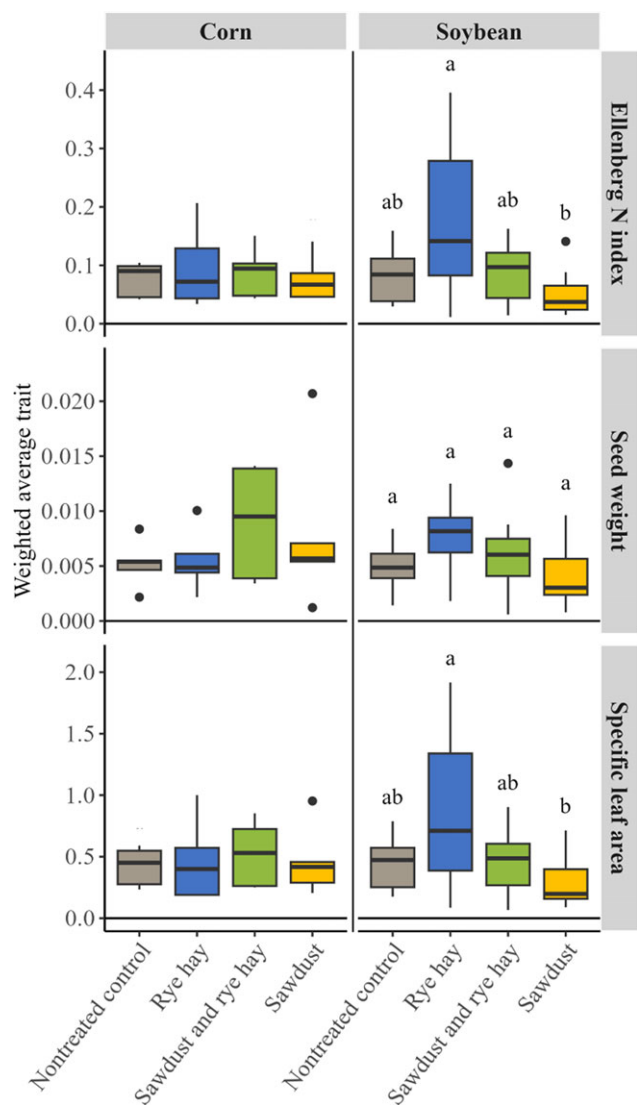
Figure 2. Average weed species traits in plots treated with different amendments for corn and soybean plots. Trait values were retrieved from the literature for each species and weighted by the number of that species found in a plot. Box plots labeled with different letters indicate significantly different means (P < 0.05) by treatment. Average weed species traits not significantly affected by soil amendments are not labeled with letters.

INDICSPECIES v. 1.7.12) based on weed biomass per species per plot, excluding weed-free control plots. To assess weed species traits, representative Ellenberg N index values, seed masses, and specific leaf areas were obtained for each weed species in our data set. Data were preferentially taken from Bàrberi et al. (2018), then from the TRY Plant Trait Database (Kattge et al. 2020), then from Mohler et al. (2021). In Mohler et al. (2021), the Ellenberg N index was not reported but responsiveness to nutrients was categorized as low, moderate, or high. Ellenberg N indexes of 2, 5, and 8 were assigned to those categories, respectively. Specific leaf area of large crabgrass [*Digitaria sanguinalis* (L.) Scop.] was taken from Garnier et al. (1997). Representative trait values for each species were multiplied by the number of individuals found in each plot. The resulting values were summed across all weed species, then divided by the total number of weeds found in the plot to calculate the weighted mean trait value for the weed community. Weighted mean trait values were used as dependent variables for linear mixed-effects models, as described earlier.

Soil respiration, nitrate, and ammonium were analyzed for each crop separately. For corn treatments, the amendment was the only fixed effect. For soybean treatments, the fixed effects were amendment, inoculation, and the interaction between amendment and inoculation. Post hoc analyses were performed using Tukey's HSD (function *cld*, in package LSMEANS v. 2.27-62). Individual soil health indicator test values from the Cornell Soil Health Test were rated on a scale of 0 to 100 based on their desirability (Moebius-Clune et al. 2016). These ratings were averaged across all indicator tests to create the overall soil health indicator score. This overall soil health score and the raw values (not rated on the 0 to 100 scale) were analyzed using linear mixed-effects models, as described earlier. Raw values were also centered and scaled, and a principal component analysis (PCA) was run (function *prcomp*, in package STATS v. 3.6.2). The first two eigenvectors were plotted to show the dominant relationship between the plots. The rotation data were plotted to show the influence of each soil health indicator test on the overall soil health score.

To prepare data for microbial community analyses, samples with fewer than 1,000 reads were removed. Data were randomly subsampled to give an equal number of reads per sample (function *rrarefy*, in package VEGAN v. 2.6-4), and feature counts were converted to percentages. Percent abundance was converted to a Bray-Curtis distance matrix (function *vegdist*, in package VEGAN v. 2.5-7). A principal coordinates analysis (PCoA) was performed on the distance matrix (function *cmdscale*, in package STATS v. 4.1.2). A permutational multivariate analysis of variance (PERMANOVA) using 999 permutations was run to determine significant differences between treatments with pseudo P-values (function *adonis2*, in package VEGAN v. 2.5-7).

Differential abundances of OTUs based on rye or no-rye amendment were calculated in two ways. The first workflow used a Dirichlet-multinomial model with transformed (centered log-ratio transformation) nonrarefied abundance data (function *aldex*, in package ALDEx2 v. 1.4.0) (Fernandes et al. 2014). The second workflow detected multivariable associations using linear models of normalized (total-sum scaling) and log-transformed non-rarefied abundance data with a Benjamini-Hochberg correction method to reduce the false discovery rate (function *Maaslin2*, in package MAASLIN2 v. 1.8.0) (Mallick et al. 2021). Additionally, a random forest machine learning algorithm was used (function *randomForest*, in package RANDOMFOREST v. 4.6-14) to create a predictive model of rye-amended versus no-rye soils. Random

and the interaction of inoculation and amendment. Block was always included as a random effect. The correlation between soybean seed yield and soybean aboveground biomass was calculated using the Spearman method. The same fixed and random effects, except weed biomass, were included in models of total weed biomass across all plots. The crop biomass per meter of crop row and the weed biomass per square meter were summed, and then percent crop biomass of total biomass was calculated. These weed and crop biomass data were collected at different times in the growing season, so the percent crop biomass measure is intended as a coarse way to compare the relative growth of crop and weeds but is not a measure of the carrying capacity of the soil. The crop biomass, weed biomass, and percent crop biomass of total plant growth were analyzed separately for each year, using a linear mixed-effects model that included all the aforementioned covariates except year.

We analyzed the weed community composition, using an indicator species analysis (function *multipatt*, in package
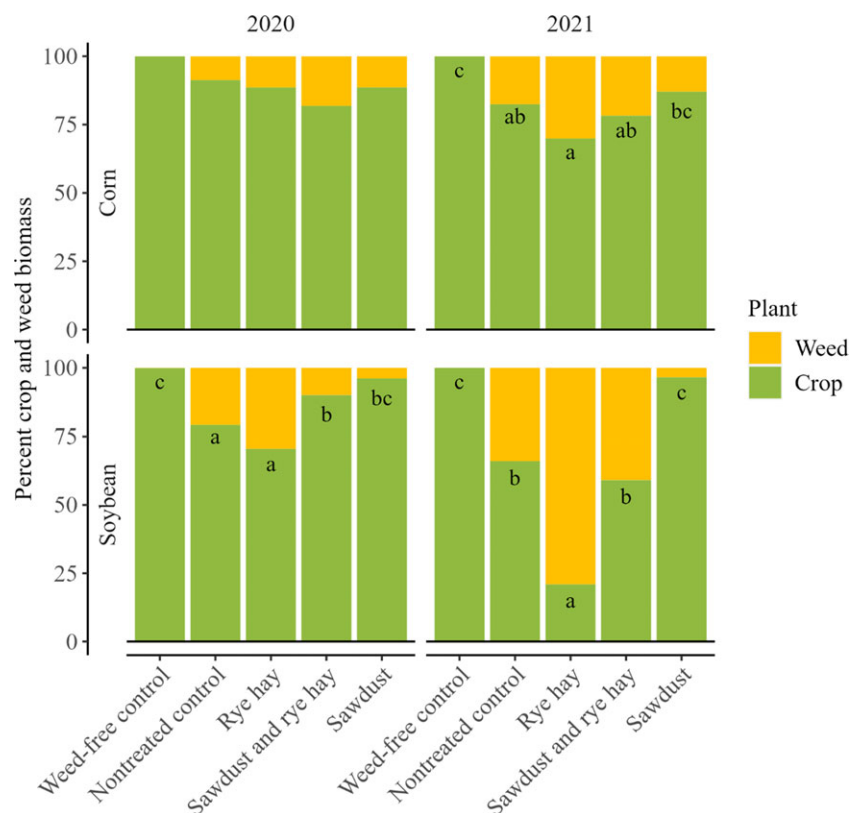
Figure 3. Percent soybean and corn crop yield relative to the total aboveground plant biomass within a square meter in soils with different amendments. Bars labeled with different letters indicate significantly different means (P < 0.05) by treatment. Years where percent plant biomass was not significantly affected by soil amendments are not labeled with letters.

forest was run on centered log-ratio values of OTUs with greater than 2% abundance across all the plots.

Results and Discussion

Amendments with High C:N Shift the Competitive Ability of Soybeans over the Weed Community

Soybean Biomass Changed with Soil Amendments

Soybean aboveground biomass was affected by soil amendment treatments (df = 4, F-value = 6.97, P < 0.0001) and year (df = 1, F-value = 6.85, P = 0.01) (Table 2) and was highly correlated with seed yield (R = 0.98). Inoculation treatment, total weed biomass, and their interactions did not affect soybean biomass. All amendment treatments reduced aboveground biomass compared with the weed-free control, except sawdust and rye hay combined in 2020 (df = 4, F-value = 10.06, P < 0.0001) (Figure 1). In 2020 and 2021, the nontreated control and the rye hay–amended soils had the lowest soybean biomass, respectively.

Total Weed Biomass and Weed Species Community Changed with Soil Amendments

Total weed aboveground biomass was also affected by soil amendment treatments (df = 4, F-value = 146.6, P < 0.0001) (Table 2). Rye hay–amended plots consistently had the highest weed biomass. Sawdust-amended plots and sawdust and rye hay combined plots in 2020 did not have higher weed biomass than the weed-free control plots. Rye hay is notable for containing benzoxazinone, which is an allelopathic compound (Du Fall and Solomon 2011; Schulz et al. 2013). This study did not explore

allelopathy, and so it is outside the realm of this discussion. However, we do not believe that allelopathy played a strong role in weed suppression in this experiment, as rye hay–amended plots consistently had the highest weed growth.

Soil Amendments with Lower C:N Ratios Selected for Nitrogen-Responsive Weed Communities

In addition to total weed biomass, the weed community composition changed based on the soil amendment treatments in soybean plots. An indicator species analysis identified several species indicative of soil amendment treatments in 2020 and 2021 (Table 3). These species were identified as having a high specificity, which is a measure of how predictive a species is of a treatment, and/or a high sensitivity of a species to a treatment, which is the estimate of the probability of finding the species in sites belonging to the site group (de Cáceres and Legendre 2009; Dufrêne and Legendre 1997). More weed species were identified as indicator species in 2021 than in 2020, suggesting greater species differentiation over time. In 2020, common purslane (*Portulaca oleracea* L.) had a specificity value of 1.0, indicating it was only found in nontreated control, rye hay, and sawdust and rye hay plots (not sawdust-amended plots). Only *Amaranthus* spp. were found in all rye hay–amended plots, but all identified species were indicative of rye hay–amended plots. In 2021, this trend held; all identified species were indicative of rye hay–amended plots, but *Amaranthus* spp. and common lambsquarters (*Chenopodium album* L.) were indicative of only rye hay–amended plots (but were not found in every rye hay–amended plot). Common chickweed [*Stellaria media* (L.) Vill.] was only found in rye hay and sawdust and rye hay
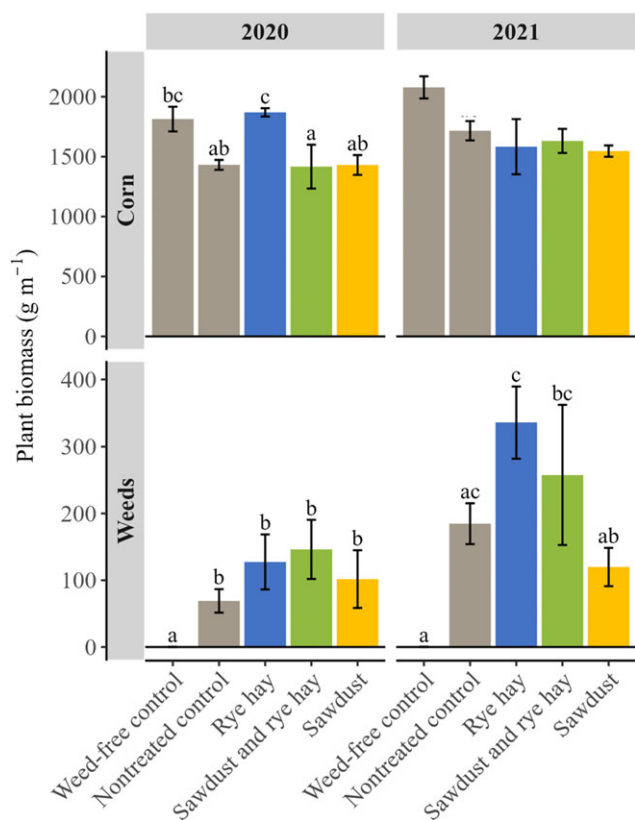
Figure 4. Corn crop yield and total weed biomass in soils with different amendments. Bars labeled with different letters indicate significantly different means (P < 0.05) by treatment. Years where total plant biomass was not significantly affected by soil amendments are not labeled with letters.

combined plots. Both witchgrass (*Panicum capillare* L.) and hairy nightshade (*Solanum physalifolium* Rusby) were only found in nontreated control, rye hay, and sawdust and rye hay combined plots (not sawdust-amended plots).

Along with a change in weed species composition, there was a corresponding change in weed species traits. In 2021, weighted-average weed species traits were affected by soil amendment treatment (Figure 2). The Ellenberg N index (df = 3, F-value = 7.17, P = 0.0007), seed weight (df = 3, F-value = 3.26, P = 0.03), and specific leaf area (df = 3, F-value = 6.14, P = 0.002) all showed a similar trend with the highest value in rye hay–amended plots and the lowest value in sawdust-amended plots, although no treatments were significantly different according to post hoc analysis of seed weight.

Taken together, these data suggest that rye hay, which had a low C:N, quickly selected for specific weed communities. Many of these species, such as *Amaranthus* spp. and *C. album*, are highly responsive to nitrogen (Blackshaw et al. 2003; Costea et al. 2004; Lindsey et al. 2013), and the whole weed community was selected for functional traits associated with r-selected growth: aboveground resource capture and nitrogen responsiveness (Grime 1977; Grime and Hunt 1975; Moreau et al. 2014). The trend toward higher seed weight in rye hay–amended soils was unexpected, because r-selected species are often associated with smaller seed weights (Booth et al. 2003; Tilman 1985). Low weighted-average Ellenberg N index values and low specific leaf area suggested some corresponding selection of species associated with K-selected growth in sawdust-amended soils,

which had the highest C:N. However, selection of K-selected species may be slower, as no indicator species were identified for sawdust-amended plots.

In High Carbon-amended Soils, Percent Soybean Aboveground Biomass Increased

These changes in soybean aboveground biomass and weed community composition and biomass resulted in significant changes in percent soybean biomass of total plant growth measured within a plot based on soil amendment treatments. This percentage does not represent the carrying capacity of the soil, as weed and crop biomass was collected at different times, but may provide an indication of the relative growth of crops and weeds. As the C:N of amendments increased, there was a greater proportion of soybean biomass in both 2020 (df = 4, F-value = 25.96, P < 0.0001) and 2021 (df = 4, F-value = 47.89, P < 0.0001) (Figure 3). This effect was driven both by higher soybean biomass in soils amended with higher C:N amendments, as well as lower weed biomass. Conversely, soils amended with lower C:N amendments always had the highest percentage of weed growth, which was correlated with the shift in weed community traits toward greater response to nitrogen and resource capture.

These data suggest that soybeans had an increased competitive advantage over weeds in soils with high C:N amendments, which is consistent with studies assessing soybean yield in cover crop systems within soils with low available nitrogen (Pittman et al. 2020; Wells et al. 2013; Williams et al. 2018). Soybeans can fix up to 300 kg N ha^{-1} or 95% of their own nitrogen from the atmosphere by forming a symbiotic relationship with rhizobia in the soil (Keyser and Li 1992; Mourtzinis et al. 2018). This trait can confer a competitive advantage to soybeans over weeds, especially in low-resource soils (van Heemst 1985). In our study, soil amendment treatment (df = 4, F-value = 2.87, P-value = 0.03), but not rhizobial inoculation (df = 1, F-value = 0.004, P-value = 0.95), affected nodulation rates. The sawdust and rye hay combined treatment had the highest nodulation rates, and weed-free control plots had the lowest nodulation rates. This pattern was correlated more with soil respiration and percent soil carbon than with nitrogen availability. Soil respiration and percent soil carbon were also highest for the sawdust and rye hay combined plots and lowest in the weed-free control plots. Many factors affect biological nitrogen fixation, but it is well established that nitrogen fixation increases as soil available nitrogen rates decrease (Keyser and Li 1992; Siczek and Lipiec 2011). From our data, it is likely that both percent soil carbon and soil nitrogen availability could play a role in modulating soybean nodulation rates.

Amendments Did Not Shift the Competitive Ability of Corn

Corn Biomass Did Not Change with Soil Amendments

Total corn biomass was not significantly affected by any independent variable (Table 2) except when analyzed separately by year. Sawdust and rye hay combined plots reduced corn biomass relative to the weed-free control in 2020 (df = 4, F-value = 5.28, p = 0.007) (Figure 4). However, total weed biomass in corn plots followed the same trend as weeds in soybean plots, consistently varying based on amendment treatment (df = 4, F-value = 68.3, P < 0.0001). In 2020, all amendment treatments had increased weed biomass compared with the weed-free control, but in 2021, only the rye hay and sawdust and rye hay combined plots had higher weed biomass than the weed-free control.

Table 4. Mean soil measurements (respiration, nitrate, and ammonium) in soils with different amendment treatments[a]

			Weed-free control	Nontreated control	Rye hay	Sawdust and rye hay	Sawdust
	Respiration				ppm CO_2 s^{-1}		
Soybean	2020	July	2.75 a	3.49 a	4.27 a	7.16 b	5.35 ab
		August	2.62 a	3.32 ab	3.77 ab	5.62 c	4.99 bc
	2021	June	1.71	2.67	2.46	3.55	2.90
		July	3.40 a	3.51 a	9.14 a	8.57 a	5.14 a
		August	6.06 a	8.10 a	10.51 a	12.59 a	8.86 a
		September	3.26 abc	2.98 ab	5.39 bc	7.51 c	2.79 a
Corn	2020	July	2.18 a	2.15 a	2.40 a	4.70 b	2.61 ab
		August	1.56 a	1.41 a	1.77 ab	2.25 ab	2.61 b
	2021	June	1.48 a	1.47 a	2.72 bc	3.84 c	2.29 ab
		July	1.81 a	3.24 a	16.24 ab	27.34 b	4.46 a
		August	5.72 a	6.33 a	12.31 ab	16.94 b	5.97 a
		September	4.98	3.48	6.01	9.89	4.44
	Nitrate				ppm NO_3^--N w^{-1}		
Soybean	2020	July	0.057 a	0.055 a	0.155 b	0.077 a	0.018 a
		August	0.042	0.037	0.079	0.098	0.026
	2021	June	0.075	0.040	0.059	0.056	0.007
		July	0.011 b	0.004 a	0.004 a	0.003 a	0.001 a
		August	0.017 b	0.008 ab	0.013 ab	0.007 a	0.006 a
		September	0.071 b	0.033 a	0.037 ab	0.013 a	0.022 a
Corn	2020	July	0.285	0.171	0.399	0.202	0.057
		August	0.058 bc	0.029 ab	0.066 a	0.049 abc	0.014 a
	2021	June	0.091 b	0.046 ab	0.082 ab	0.016 ab	0.007 a
		July	0.009 b	0.004 ab	0.006 ab	0.005 ab	0.002 a
		August	0.010	0.003	0.008	0.007	0.004
		September	0.012	0.008	0.009	0.011	0.004
	Ammonium				ppm NH_4^+-N w^{-1}		
Soybean	2020	July	0.0001 a	0.0003 a	0.0007 b	0.0004 a	0.0002 a
		August	0.0004	0.0006	0.0012	0.0004	0.0002
	2021	June	0.0004	0.0005	0.0005	0.0007	0.0005
		July	0.0002	0.0002	0.0008	0.0003	0.0004
		August	0.0007	0.0006	0.0011	0.0008	0.0012
		September	0.0013	0.0032	0.0031	0.0028	0.0012
Corn	2020	July	0.0033	0.0008	0.0030	0.0033	0.0008
		August	0.0005	0.0010	0.0020	0.0010	0.0002
	2021	June	0.0004 a	0.0004 a	0.0015 b	0.0004 a	0.0002 a
		July	0.0002	0.0004	0.0006	0.0002	0.0007
		August	0.0010	0.0007	0.0008	0.0017	0.0005
		September	0.0007	0.0007	0.0017	0.0008	0.0010

[a]Different letters indicate significantly different means (P < 0.05) by treatment for that sample date.

Weed Species Community Composition Was Minimally Changed with Soil Amendments

Despite changes in total weed biomass, there was very little evidence for changes in weed community composition based on the soil amendment treatments in corn plots. An indicator species analysis did identify several species indicative of soil amendment treatments in 2020 but none in 2021 (Table 3). In 2020, *D. sanguinalis* was found in all sawdust and sawdust and rye hay combined plots. Both shepherd's purse [*Capsella bursa-pastoris* (L.) Medik.] and *P. oleracea* followed the same trends in corn plots as they did in soybean plots. *Capsella bursa-pastoris* was indicative of nontreated control plots and rye hay–amended plots. *Portulaca oleracea* was indicative of nontreated control plots, rye hay plots, and sawdust and rye hay combined plots (not sawdust-amended plots). Mirroring this minor change in weed species composition, there was also no change in weed species traits (Figure 2).

Reductions in Total Weed Biomass Drove Changes in Percent Corn Aboveground Biomass

Percent corn biomass of total plant growth was not affected by soil amendments in 2020 (df = 4, *F*-value = 2.88, P = 0.06), but it was in 2021 (df = 4, *F*-value = 8.36, P = 0.0008) (Figure 3). Weed-free control plots all had the highest percentage of crop growth,

followed by sawdust-amended plots. Rye hay–amended plots consistently had the lowest percentage of crop growth. These data suggest that higher C:N amendments did not increase the competitiveness of corn relative to weeds. As in soybean, the percent corn biomass of the total plant community increased with higher C:N amendments. However, this trend was driven by the changing biomass of the weed community, not changes in weed community composition or in corn growth. Similar to soybean plots, total weed growth decreased in soils with higher C:N amendments, but unlike soybean, corn biomass did not vary based on the amendment.

Only one cornfield and one soybean field were included in our study, so we cannot statically compare the two crops. However, we were able to find evidence that soybeans became more competitive with weeds as soil was subjected to higher C:N amendments, but this trend was not found for corn. Crop type can be one of the strongest filters on weed community composition (Smith and Gross 2007). This has been observed for different species included in intercropped systems (Gomez and Gurevitch 1998) and even for different cultivars of the same crop (de la Fuente et al. 2006). However, this filter has often been attributed to differences in cultural management practices such as crop rotations (Sosnoskie et al. 2006) or cover cropping (Smith and Gross 2007). Within our
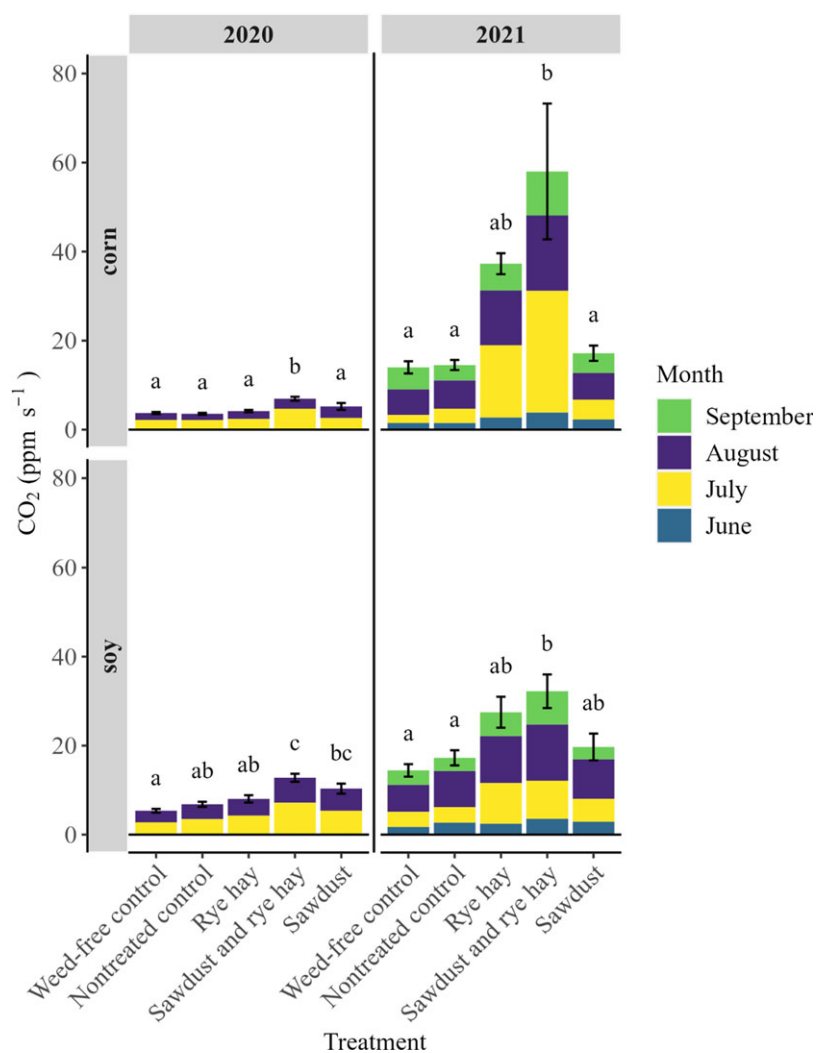
Figure 5. Mean total soil respiration as affected by amendment treatment in 2020 and 2021. Measurements were taken twice in 2020 (July and August) and four times in 2021 (June, July, August, and September). Bars labeled with different letters indicate significantly different means (P < 0.05) by treatment within year.

study, most management practices were the same for both corn and soybean plots—field preparation, planting, harvesting, irrigation, and data collection were all done at approximately the same time. However, we did band-fertilize corn at planting, which may have reduced differences in crop growth and/or provided space for nitrogen-responsive weeds to grow in plots where nitrogen was otherwise immobilized. Unfortunately, we did not measure between-row and within-row differences in nitrogen availability or weed community, so we cannot confirm whether this was the case. There was also a difference in planting spacing. Rates were chosen based on local optimal spacing, but the denser seeding rate for soybeans may have resulted in greater changes in the weed community composition. Findings from other studies have shown stronger selection pressure on the weed community in soybean fields compared with cornfields (Culpepper 2006; Rauber et al. 2018).

These findings highlight the potential to utilize reverse fertilization in agriculture. In a highly fertilized field, nitrogen-responsive weeds will eventually dominate the weed community. Reverse fertilization temporarily limits nitrogen availability, reducing the competitive advantage of these weeds. Before the weed community has time to shift toward species with greater belowground investment, leguminous crops will have a competitive advantage. This competitive advantage may not occur in nonleguminous crops. However, additional research is needed to determine what effect, if any, band-fertilizing corn in our study had on results.

Indicators of Nitrogen Immobilization in Soils Amended with High C:N Amendments

Soil CO_2 was responsive to soil amendment treatments at every sample date except June (soybean soils) and September (corn soils) in 2021 (Table 4). Total respiration pooled across sample dates within a year was also different based on amendment treatment for both corn (df = 5, F-value = 10.4, P = 0.0002 in 2020; df = 5, F-value = 7.6, P = 0.0007 in 2021) and soybean (df = 6, F-value = 13.9, P > 0.0001 in 2020; df = 6, F-value = 8.4, P > 0.0001 in 2021). Respiration was consistently highest for the soil amended with sawdust and rye hay combined (Figure 5), although it was not significantly different from rye hay–amended soil for corn in 2021, sawdust-amended soil for soybean in 2020, or rye hay– or sawdust-amended soil for soybean in 2021. Soil CO_2 is a measure of microbial respiration,
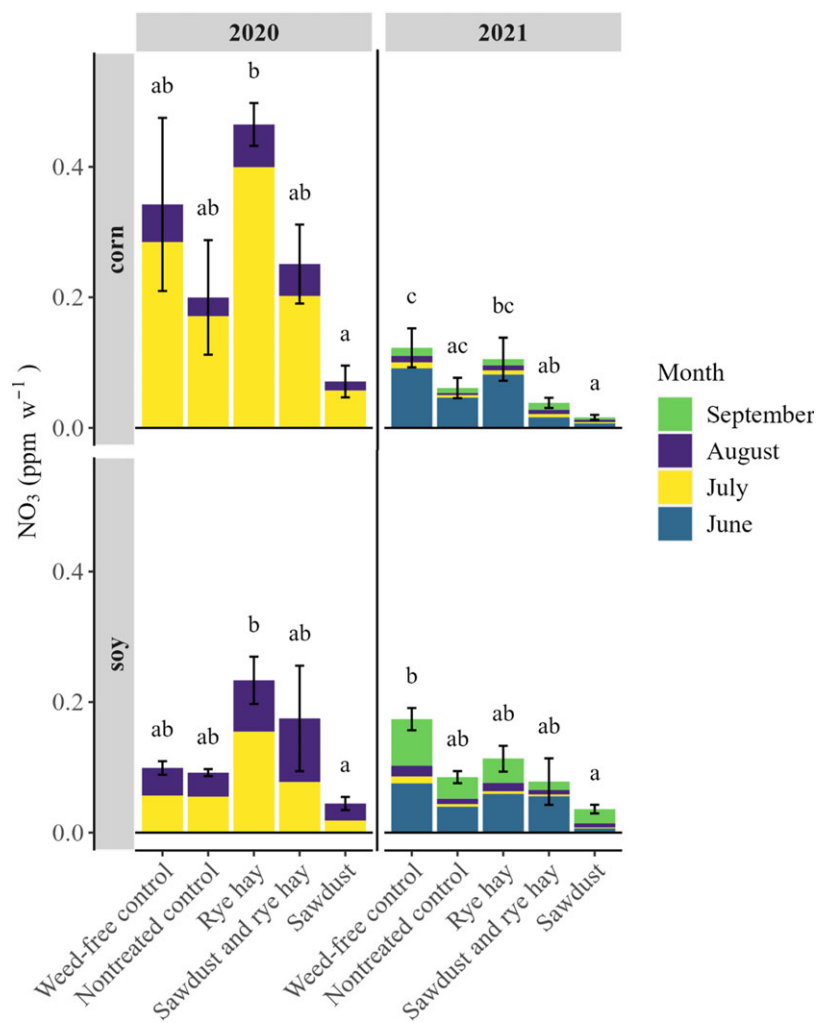
Figure 6. Mean total nitrate as affected by amendment treatment in 2020 and 2021. Measurements were taken twice in 2020 (July and August) and four times in 2021 (June, July, August, and September). Bars labeled with different letters indicate significantly different means (P < 0.05) by treatment within year.

which is an indicator of soil microbial activity. These consistently high respiration rates in sawdust and rye hay combined plots, which had the greatest total amount of carbon added to the soil, implies that carbon additions are the primary driver of microbial community activity.

Increased microbial respiration did not always correspond with decreased nitrogen availability. Total nitrate measured over each year was significantly different based on amendment treatment for both corn (df = 5, *F*-value = 3.7, P = 0.03 in 2020; df = 5, *F*-value = 4.9, P = 0.009 in 2021) and soybean (df = 6, *F*-value = 3.5, P > 0.01 in 2020; df = 6, *F*-value = 6.2, P = 0.0004 in 2021). Consistently, total nitrate was lowest in sawdust-amended soil and highest in rye hay–amended soil or unamended weed-free control soil (Figure 6). Total ammonium availability was more variable. Total ammonium was significantly affected by amendment treatment in corn in 2021 (df = 5, *F*-value = 5.5, P = 0.005) and soybean in 2020 (df = 6, *F*-value = 4.6, P = 0.003). It was numerically lowest in sawdust-amended soil in 2020, but it was numerically lowest in the nontreated control for corn and in the unamended weed-free control for soybean in 2021. Rye hay–amended soil consistently had the highest ammonium availability, although it was only significantly higher for corn in 2021 and soybean in 2020 (Figure 7).

Although less responsive than soil respiration, both nitrate and ammonium availability were lowest in sawdust-amended plots, indicating that the greatest nitrogen immobilization occurred with the highest C:N amendment. Sawdust and rye hay combined plots, which had the greatest total amount of carbon added to the soil, had greater microbial activity, as indicated by the highest soil respiration rates, but the highest respiration did not correspond to the lowest nitrogen availability. These results suggest that to create a nutrient-limiting environment, it is more important to add proportionally more carbon than nitrogen to the soil. Otherwise, the soil microbes can utilize nitrogen from the amendment source, thus leaving more nitrogen available in the soil. These results are consistent with the literature. Nitrogen immobilization is a microbially mediated process, occurring when nitrogen is replaced by carbon as the limiting nutrient for microbial community growth during substrate decomposition (Barrett and Burke 2000; Manzoni et al. 2008). It is well established that a substrate's C:N is a critical determinant of the rate and duration of nitrogen immobilization or mineralization processes (Hodge et al. 2000; Manzoni et al. 2010), although there are exceptions to this trend (Hättenschwiler et al. 2011).

Future research should focus on how to refine nitrogen immobilization into a precise reverse fertilization tool. In the
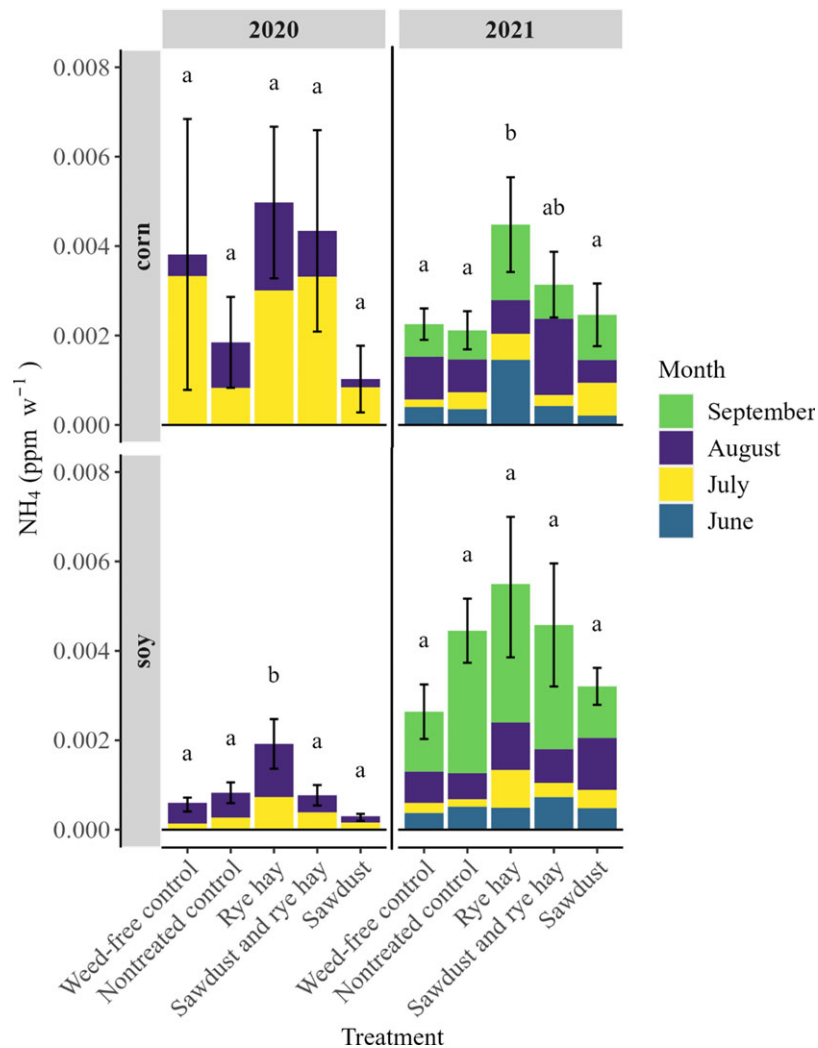
Figure 7. Mean total ammonium as affected by amendment treatment in 2020 and 2021. Measurements were taken twice in 2020 (July and August) and four times in 2021 (June, July, August, and September). Bars labeled with different letters indicate significantly different means (P < 0.05) by treatment within year.

current study, large quantities of amendments were added to the soil to markedly increase soil carbon, with little regard for decomposition rates. Reverse fertilization, however, may be more effective if little to no nitrogen is available for plants early in the growing season and the soil quickly transitions to nitrogen mineralization once crops have become established. The period of immobilization should correspond with the duration of time that nutrient resources are available in the crop seed, allowing the crop to establish dominance over the weed community before soil nitrogen becomes more readily available. This time frame would be crop specific and may be similar to the critical period of weed control. Understanding immobilization and mineralization rates as well as amendment traits will be crucial to selecting the ideal amendment for each cropping system. Many studies have suggested that organic matter characteristics, in addition to C:N, affect the rate and duration of nitrogen immobilization or mineralization. Lignin content (Aber and Melillo 1982) and solid-state ^{13}C NMR spectroscopy (Bonanomi et al. 2013) have been found to be useful in predicting substrate decomposition rates. Bonanomi et al. (2019) suggest a framework dividing amendments into four quadrants based on two scales: carbon complexity/availability and nitrogen

content. High C:N amendments with low-complexity carbon may be more appropriate for reverse fertilization.

Microbial Community Composition Was More Closely Linked to Soil Amendments Than to Soil Health Measurements

Carbon Amendment Drives Improvements in Soil Health

One of the main tenets of the soil health paradigm is to recognize the living component of soil and the important functions of soil biota in nutrient retention and cycling (Chaparro et al. 2012; Haney et al. 2018; Stika 2013). Therefore, stimulating microbial growth and nitrogen immobilization with high-carbon amend-ments may also have multifunctional benefits for improved soil health. Average soil health indicator scores in our study were affected by soil amendment treatment (df = 3, *F*-value = 5.37, P = 0.003). Sawdust and rye hay combined plots had the highest average soil health scores, followed by sawdust-amended plots, both of which had a high amount of carbon added to the soil (Figure 8A). Unamended control plots had the lowest average soil health scores. Most individual soil health indicator tests contributed to this trend. All individual soil health indicators were significantly affected by soil amendment treatment, except depth to hardpan, pH, aluminum, iron, and sulfur (Table 5). PCA
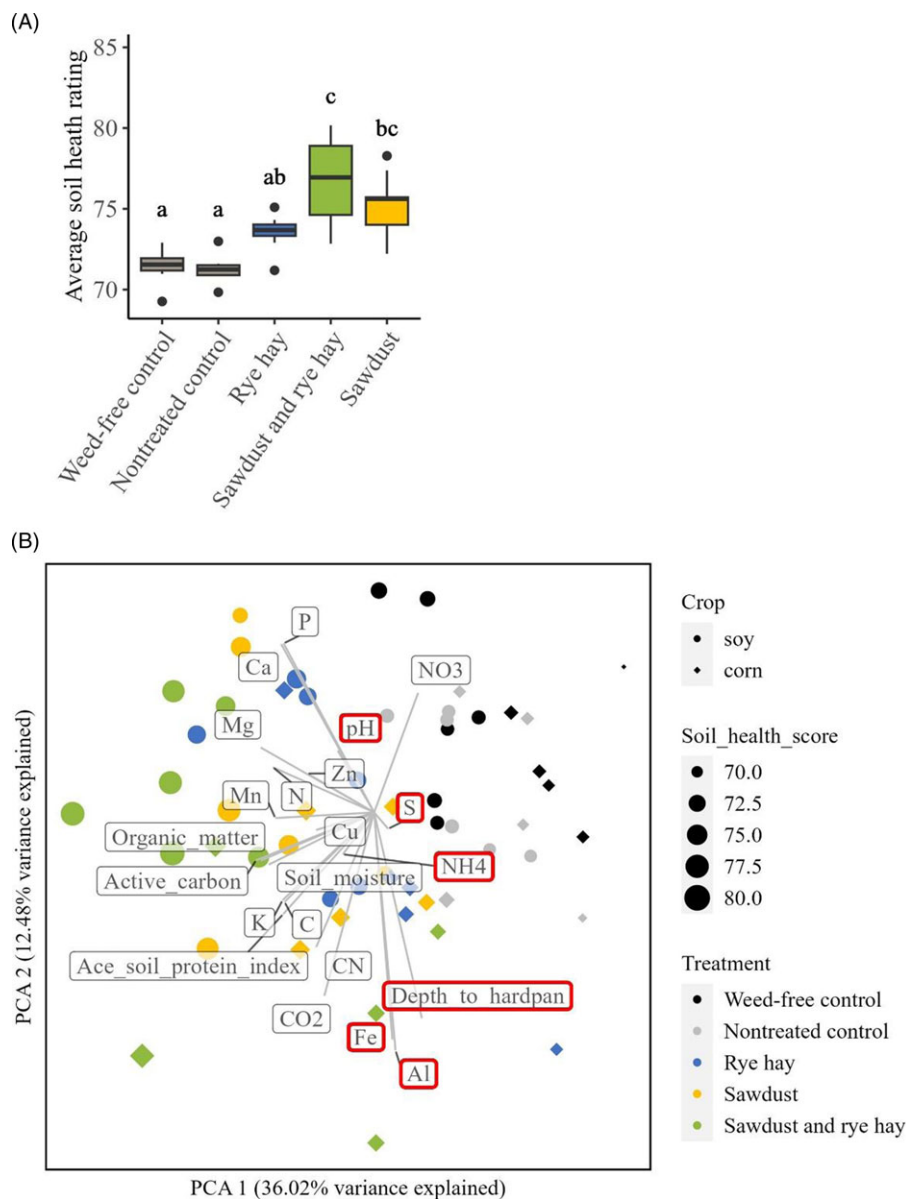
Figure 8. (A) Average soil health ratings for each plot treated with different amendments. Box plots labeled with different letters indicate significantly different means (P < 0.05) by treatment. (B) Principal component analysis (PCA) of centered and scaled soil health indicator values. Shape indicates crop, size indicates soil health rating, and color indicates amendment treatment. All indicators were significantly affected by treatment (P < 0.05), except indicators outlined in red. ACE, autoclaved-citrate extractable soil protein index.

of centered and scaled values of soil health indicator tests demonstrates that the improved health in sawdust and rye hay combined plots cluster together (Figure 8B) and are associated with several biological indicators: organic matter, active carbon, ACE soil protein, percent carbon, and soil respiration. Organic matter, active carbon, and percent carbon are primarily measures of soil carbon. Many biological soil health indicators are measures of soil carbon and carbon cycling (Bünemann et al. 2018; Liptzin et al. 2022), because soil carbon is the primary driver of the soil food system and is therefore critically important in increasing soil health (Lal 2014, 2016). Active carbon is a measure of the potassium permanganate oxidizable carbon, which is a proxy for the food source of microbial communities (Weil et al. 2003). The ACE soil protein index estimates the amount of organically bound, or

bioavailable, nitrogen by measuring a range of proteins extracted from soil (Hurisso et al. 2018).

Improved soil health ratings in our study were also associated with chemical soil health indicators: phosphorus, potassium, calcium, copper, magnesium, manganese, and zinc. Increases in macro- and micronutrient availability may have come from the added sawdust or rye hay (Sadeghpour et al. 2021) or increased microbial activity may have improved access to soil nutrients (Song et al. 2019). Increased soil moisture was also associated with improved average soil health ratings. Soil moisture may have increased due to the increased water-holding capacity in soils with added organic matter (Thangarajan et al. 2013). These improved soil biological, chemical, and physical health indicators were well correlated with the amount of total carbon added to the soil.

Table 5. The P-value and mean soil health indicator value by soil amendment treatment[a]

	P-value	Weed-free control	Untreated control	Rye hay	Sawdust and rye hay	Sawdust
Organic matter %	<0.0001	2.14 a	2.15 a	2.34 a	2.71 b	2.7 b
Soil respiration ppm s^{-1}	<0.0001	15.1 a	16.6 a	34.5 bc	46.3 c	18.2 ab
Active carbon mg kg soil^{-1}	<0.0001	360 a	347 a	417 b	466 b	446 b
Carbon %	0.0005	1.08 a	1.17 a	1.25 a	1.51 b	1.53 ab
Carbon:nitrogen ratio	0.0002	9.25 a	9.8 ab	9.39 ab	11.47 b	12.12 ab
Nitrogen %	<0.0001	0.116 a	0.119 ab	0.133 b	0.132 ab	0.126 b
NO$_3^-$ ppm w^{-1}	<0.0001	0.150 c	0.074 abc	0.101 bc	0.039 ab	0.032 a
ACE soil protein index[b] mg g soil^{-1}	<0.0001	3.62 b	3.61 ab	4.16 abc	4.43 c	4 bc
Soil moisture %	<0.0001	15.6 a	16 ab	16.5 ab	18.1 c	17.7 bc
Phosphorous mg kg soil^{-1}	0.04	13.1 a	12.6 a	14.4 a	16.2 a	15.2 a
Potassium mg kg soil^{-1}	<0.0001	104 a	116 ab	204 c	248 d	142 b
Calcium mg kg soil^{-1}	0.05	924 a	886 a	950 a	982 a	1013 a
Copper mg kg soil^{-1}	0.03	0.119 b	0.119 ab	0.126 ab	0.125 ab	0.134 a
Magnesium mg kg soil^{-1}	<0.0001	60.8 a	61.1 ab	67.8 abc	72 bc	70.4 c
Manganese mg kg soil^{-1}	<0.0001	1.47 a	1.54 a	2.17 ab	2.57 b	2.02 ab
Zinc mg kg soil^{-1}	0.01	0.115 a	0.107 a	0.150 a	0.150 a	0.150 a

[a]Values labeled with different letters indicate significantly different means from other values within the row based on Tukey's post hoc analysis.
[b]Autoclaved-citrate extractable (ACE) soil protein index.

Microbial Community Composition Was Most Closely Linked to Rye Hay Amendments

In both corn and soybean plots, microbial beta diversity clustered by treatment in a PCoA (Figure 9). The PERMANOVA results indicate significant associations between the microbial community and year (P-value = 0.001 for bacteria; P-value = 0.001 for fungi), soil amendment treatment (P-value = 0.001 for bacteria; P-value = 0.001 for fungi), and crop (P-value = 0.005 for bacteria; P-value = 0.001 for fungi). When beta diversity is analyzed separately for each year, or for only the soils where soil health was measured, soil amendment treatment and crop remain significant (Table 6).

Community composition clustered most strongly around the rye hay amendment. Comparisons between rye hay–amended plots (including both rye hay plots and sawdust and rye hay combined plots) and no-rye plots (including both unamended controls and sawdust plots) showed clearer patterns than comparisons among all five amendment treatments. The random forest algorithm accurately divides plots into rye hay–amended or no–rye hay plots using both bacterial and fungal communities. The out of box (OOB) error rates for bacterial communities were 5.41% in 2020 and 3.33% in 2021. The OOB error rates for fungal communities were 8.24% in 2020 and 12% in 2021. These error rates are much smaller than the error rates using all five amendment treatments as categories (55.41% in 2020 and 51.67% in 2021 for bacteria; 24.71% in 2020 and 24% in 2021 for fungi).

The results from Maaslin2 support this trend: OTUs with significantly different abundance between rye-amended and no-rye treatments were identified for bacteria and fungi in both years. Percent abundances in rye and no-rye plots are shown in Figure 10 for the 10 OTUs with the lowest q-values, and taxonomic information for these 10 OTUs is presented in Table 7. Despite the low random forest model error rates and the identification of OTUs with differential abundance between rye-amended and no-rye plots according to Maaslin2, the ALDEx2 approach did not identify any bacterial or fungal OTUs as differentially abundant. ALDEx2 is more conservative in measuring differential abundance than Maaslin2; however, Maaslin2 is more conservative than other methods (Nearing et al. 2022). Combining methods (Maaslin2 and random forest) can increase confidence in the conclusion that

there is a strong difference in microbial community composition between rye and no-rye plots.

Exploring the differentially abundant OTUs based on rye amendment in our experiment, many of the microbes that were more abundant in rye-amended soils appear to be beneficial. Two strains of *Stenotrophomonas geniculata* were significantly more abundant in rye-amended soils, compared with no-rye soils, in 2020. *Stenotrophomonas geniculata* has been shown to have plant growth-promoting properties in chick pea (*Cicer arietinum* L.) nodules and corn (Gopalakrishnan et al. 2015; Singh et al. 2020). *Stenotrophomonas geniculata* can also decompose complex compounds such as the herbicide paraquat (Wu et al. 2020), nicotine (Liu et al. 2014), and polybutylene succinate/polylactic acid (Srimalanon et al. 2020). *Pseudomonas vancouverensis* was also more abundant in rye-amended soils and has been shown to improve the stress tolerance of red peppers (*Capsicum annuum* L.) (Samaddar et al. 2019) and tomatoes (*Solanum lycopersicum* L.) (Subramanian et al. 2015). An uncultured *Staphylococcus*, which was more abundant in rye hay plots than no-rye plots, was the only OTU shared between the 2020 and 2021 lists of the top 10 bacterial OTUs with significantly different abundance. In 2021, the three most significantly different bacteria were *Bacillus* species, which contribute to macronutrient cycling by solubilizing phosphorous-containing potassium compounds (Song et al. 2019). These three bacteria were more abundant in rye hay–amended soils, although the sixth most significantly different OTU was a *Bacillus* species more abundant in no-rye soils.

Two fungal OTUs were identified by Maaslin2 as significantly different between rye hay and no-rye plots in both years. Both OTUs were more abundant in rye-amended soils. *Papiliotrema laurentii* is found in a variety of habitats and can metabolize a variety of carbon substrates. It can control phytopathogenic fungi and improve mycorrhizal colonization, nitrogen retention, and plant growth (de Almeida et al. 2022). The other fungus was in the family Nectriaceae, but was not further classified. *Trichosporon insectorum*, *Ascobolus foliicola*, and *Trichoderma evansii* were all more abundant in rye-amended soils in 2021. *Trichosporon insectorum* is a yeast, often associated with insects, with fungicidal properties (Fuentefria et al. 2008). *Ascobolus foliicola* is often found on bare ground or decomposing plant matter (Uzun et al. 2019),
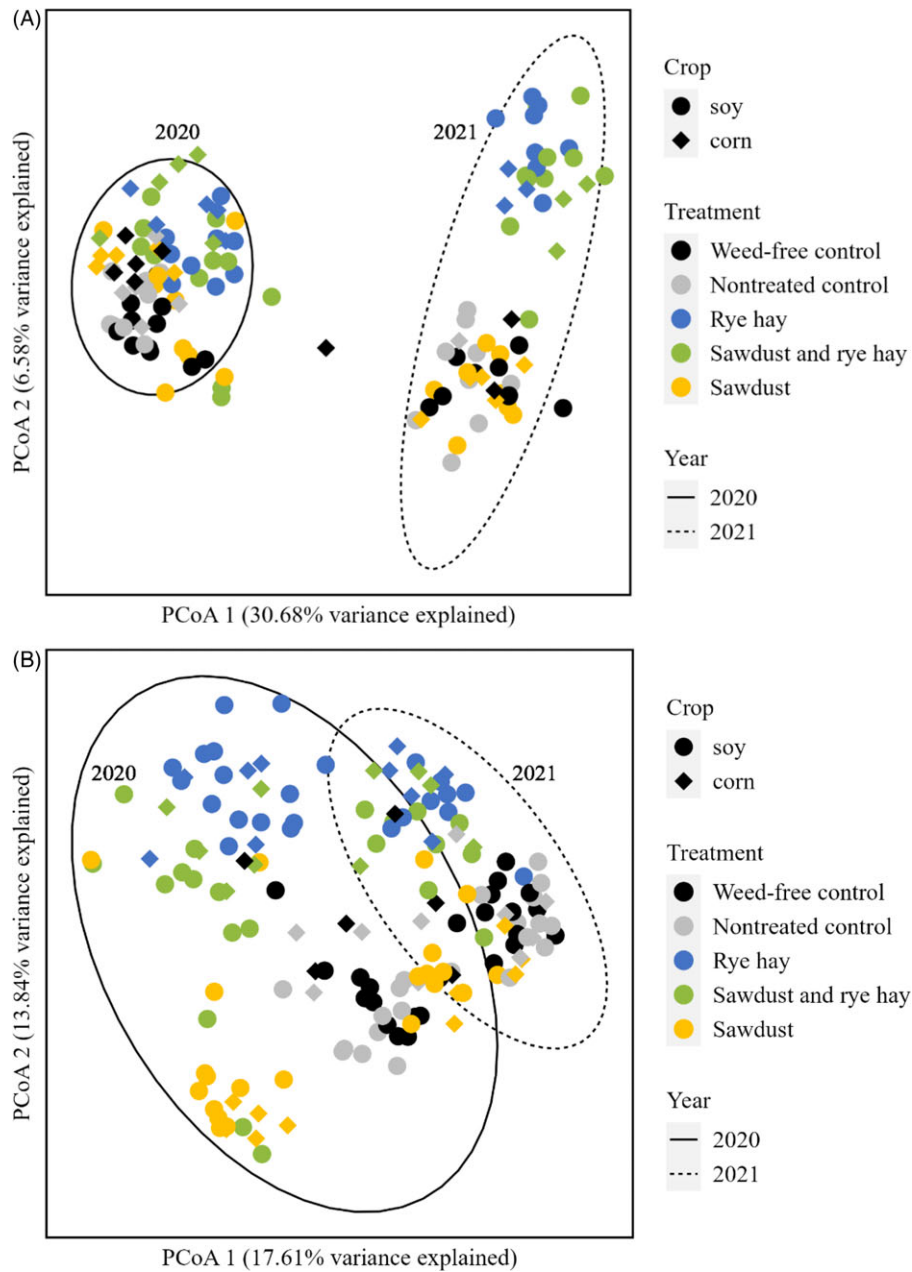
Figure 9. Principal coordinates analysis (PCoA) of the bacterial (A) and fungal (B) communities based on Illumina MiSeq amplicon sequencing. Shape indicates crop, color indicates amendment treatment, and ellipse line type indicates year. Permutational multivariate analysis of variance (PERMANOVA) revealed significant effects of soil amendment (pseudo P-value = 0.001 for bacteria and fungi), crop (pseudo P-value = 0.005 for bacteria; pseudo P-value = 0.001 for fungi), and year (pseudo P-value = 0.001 for bacteria and fungi) on microbial community composition.

and *Trichoderma evansii* was isolated from the sapwood of *Lophira alata* and cola [*Cola verticillata* (Thonn.) Stapf ex A. Chev.] (Samuels and Ismaiel 2009).

Rye hay–amended plots did seem to support the growth of beneficial microbial communities, but that change was not directly captured in the soil health indicator tests, which were higher as the total carbon amount added to the soil increased. This finding is consistent with the literature, which shows that it is often difficult to directly connect the whole soil microbiome to soil health functioning (Bünemann et al. 2018). The measures of soil biological health often included in soil health studies are primarily measures of carbon and nitrogen cycling, which are indirect measures of the soil microbial community. It is still expensive to

measure the microbial community composition directly, and more research needs to be done to link specific communities to improved soil health. A recent study by Wilhelm et al. (2022) used microbial community composition to predict soil health ratings with almost 70% accuracy. In our study, the total microbial activity was more important for improving soil health ratings and altering plant growth dynamics than the specific microbial community composition.

In summary, this study demonstrates that reverse fertilization can improve the competitive advantage of leguminous crops with multifunctional benefits for soil health. High C:N organic amendments stimulated nitrogen immobilization through microbial growth, and greater amounts of added carbon improved soil

Table 6. The P-values and degrees of freedom (df) for bacterial (16S rRNA region) and fungal (ITS2 region) beta diversity, measured with a permutational multivariate analysis of variance (PERMANOVA) on a principal coordinates analysis (PCoA) of a Bray-Curtis distance matrix of operational taxonomic units (OTUs)

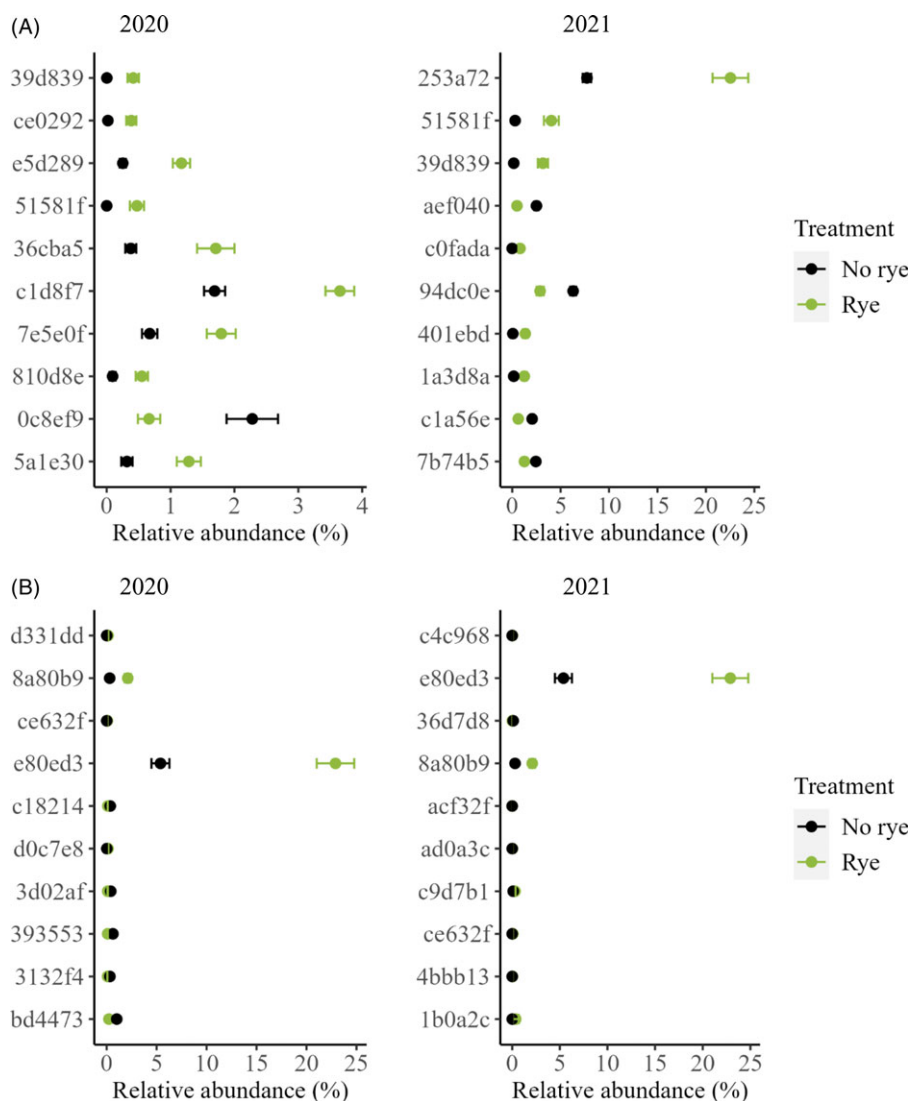|	Bacteria		Fungi	
Dependent variable	df	P-value	df	P-value
		2020		
Amendment	4	0.001	4	0.001
Crop	1	0.001	1	0.002
Inoculation	1	0.077	1	0.72
Amendment × crop	4	0.57	4	0.02
Amendment × inoculation	4	0.71	4	0.91
		2021		
Amendment	4	0.001	4	0.001
Crop	1	0.009	1	0.001
Inoculation	1	0.87	1	0.93
Amendment × crop	4	0.19	4	0.30
Amendment × inoculation	4	0.32	4	0.99
		Only soils tested for soil health		
Amendment	4	0.001	4	0.001
Crop	1	0.03	1	0.001
Amendment × crop	4	0.12	4	0.22

Figure 10. The 10 bacterial (A) and fungal (B) operational taxonomic units (OTUs) whose abundances differed most significantly between rye-amended and no-rye plots. These OTUs were identified with multivariable associations with linear models (Maaslin2). Maaslin2 was run on nonrarefied abundance data, but percentage relative abundance data are shown. Identified taxa are ordered from most (top) to least (bottom) significantly different. Full taxonomic classification for each identified taxon can be found in Table 7.

Table 7. Top 10 bacterial and fungal operational taxonomic units (OTUs) with significantly different abundance between plots amended with rye hay and no-rye plots, as identified by the Microbiome Multivariable Association with Linear Models 2.0 algorithm

OTU[a]	Phylum	Class	Order	Family	Genus	Species	p
Bacteria							
2020							
39d839	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*		7.98×10^{-11}
ce0292	Proteobacteria	Alphaproteobacteria	Hyphomicrobiales				7.15×10^{-10}
e5d289	Proteobacteria	Alphaproteobacteria	Hyphomicrobiales	Devosiaceae	*Devosia*		5.08×10^{-9}
51581f	Firmicutes	Bacilli	Bacillales	Staphlococcaceae	*Staphlococcus*		1.76×10^{-8}
36cba5	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	*geniculata*	2.65×10^{-8}
c1d8f7	Actinobacteriota	Actinomycetia	Micrococcales	Micrococcaceae			7.19×10^{-8}
7e5e0f	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*vancouverensis*	2.75×10^{-7}
810d8e	Bacteroidota	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*		3.53×10^{-7}
0c8ef9	Cyanobacteria	Cyanobacteriia	Synechococcales	Trichocoleusaceae	*Trichocoleus*	*desertorum*	4.98×10^{-7}
5a1e30	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	*geniculata*	1.51×10^{-6}
2021							
253a72	Firmicutes	Bacilli	Bacillales	Staphlococcaceae	*Staphlococcus*		9.11×10^{-17}
39d839	Firmicutes	Bacilli	Bacillales	Staphlococcaceae	*Staphlococcus*		2.02×10^{-10}
51581f	Firmicutes	Bacilli	Bacillales	Staphlococcaceae	*Staphlococcus*		1.69×10^{-10}
aef040	Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae			6.24×10^{-10}
c0fada	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*	*ginsengihumi*	5.17×10^{-9}
94dc0e	Firmicutes	Bacilli	Bacillales	Staphlococcaceae	*Staphlococcus*		9.24×10^{-9}
401ebd	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Brevibacillus*		1.59×10^{-8}
1a3d8a	Firmicutes	Bacilli	Bacillales	Thermoactinomycetaceae	*Thermoactinomyces*		3.36×10^{-7}
c1a56e	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae			1.21×10^{-6}
7b74b5	Proteobacteria	Alphaproteobacteria	Hyphomicrobiales	Hyphomicrobiacea			4.88×10^{-6}
Fungi							
2020							
d331dd	Ascomycota	Orbiliomycetes	Orbiliales	Incertae sedis	*Vermispora*	*fusarina*	6.74×10^{-25}
8a80b9	Basidiomycota	Tremellomycetes	Tremellales	Rhynchogastremataceae	*Papiliotrema*	*laurentii*	8.54×10^{-14}
ce632f	Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*		9.52×10^{-14}
e80ed3	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae			7.01×10^{-12}
c18214	Ascomycota	Leotiomycetes	Helotiales				1.37×10^{-10}
d0c7e8	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae sedis	*Candida*	*tropicalis*	1.79×10^{-9}
3d02af	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Neonectria*	*lugdunensis*	2.14×10^{-9}
393553	Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis			2.68×10^{-9}
3132f4	Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*	*amoeboidea*	3.35×10^{-9}
bd4473							4.63×10^{-9}
2021							
c4c968	Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Trichosporon*	*insectorum*	4.42×10^{-16}
e80ed3	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae			1.32×10^{-12}
8a80b9	Basidiomycota	Tremellomycetes	Tremellales	Rhynchogastremataceae	*Papiliotrema*	*laurentii*	2.77×10^{-11}
36d7d8	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	*Ganoderma*		2.39×10^{-11}
acf32f	Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	*Ascobolus*	*foliicola*	5.11×10^{-11}
ad0a3c							2.11×10^{-9}
c9d7b1	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	*evansii*	2.55×10^{-9}
ce632f	Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*		2.99×10^{-9}
4bbb13							6.10×10^{-9}
1b0a2c	Ascomycota	Sordariomycetes	Sordariales	Sordariaceae			1.39×10^{-8}

[a]The six-digit OTU identifiers correspond to the same six-digit OTU identifiers in Figure 9.

health ratings. The soil microbial community composition consistently changed when different amendments were added to the soil; however, these compositional shifts were most closely associated with the presence of rye hay. Although these results are promising, reverse fertilization requires further refinement before it can be adopted by growers. Potential ways to refine this tool include immobilizing nitrogen only in specific locations such as between the crop rows or at the soil surface to inhibit the germination of small-seeded, r-selected weeds. Alternatively, nitrogen may be immobilized only during specific times, such as within the critical period of weed control or for a few years before competitive species begin to dominate. Nitrogen immobilization is an important regulator of plant growth that should be considered when designing weed management strategies in agroecosystems.

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