Received: 1 March 2024 | Accepted: 20 October 2024

DOI: 10.1002/agj2.21729

Agronomy Journal

ORIGINAL ARTICLE

Soil Fertility and Crop Nutrition

Soil inoculation with nitrogen-fixing bacteria to supplement maize fertilizer need

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Assigned to Associate Editor Augustine Obour.

Funding information
National Institute of Food and Agriculture, Grant/Award Number: NC1200

Abstract

Nitrogen (N) is an essential plant nutrient, but low and variable plant-available N levels in agricultural soils often limit maximum grain production. The objective of this study was to determine if a free-living nitrogen-fixing bacterial inoculant (NFI) could supply biologically-fixed N as an additional N source and if this enhances maize (*Zea mays* L.) N uptake and grain yield. Maize was grown at four site-years in Illinois during 2019–2021. The NFI, a mixture of edited *Klebsiella variicola* and *Kosakonia sacchari*, was applied in furrow at planting with urea-N rates from 0 to 225 kg N ha^{-1}. Using quadratic regression models, across N rates, the NFI supplemented the fertilizer-N equivalent of 38.5 or 12.1 kg N ha^{-1} at V8 or R1, respectively. Increases in N accumulation were observed in all plant fractions, and δ^{15}N abundance measurements confirmed that some of this additional N was derived from biological N fixation. The NFI treatment increased N accumulation by an average of 4.8% and 3.7% at V8 and R1, respectively, which was the result of greater biomass, with no effect on plant N concentration. Application of NFI resulted in an average of 1.5% more kernels m^{-2} and 0.11 Mg ha^{-1} more grain yield. This work reveals that NFI can provide an additional source of N for maize production but identifies that the season-long benefit of fixed-N from an NFI is yet to be fully optimized.

Plain Language Summary

Nitrogen is often the most limiting nutrient for increased maize yields, but extra fertilizer may be lost to waterways and the air. Nitrogen-fixing bacterial inoculants can convert nitrogen from the air into a usable form in the soil near the plant roots. Yet, maize plants need nitrogen at certain times and amounts to be useful to grow and make yield. We wanted to know if a nitrogen-fixing bacteria inoculation mix can replace some reliance on fertilizer, when maize takes up this nitrogen and where it goes in the plant, and if grain yield changes. Supplying the inoculant at planting in combination with low rates of fertilizer (45–135 kg nitrogen ha^{-1}) increased maize

Abbreviations: CFU, colony forming units; CU, Champaign; NFI, nitrogen-fixing bacterial inoculant; NL, Nashville; SY, site-year.

vegetative growth, nitrogen accumulation, kernel number, and yield (on average 0.11 Mg ha^{-1} more yield) and was equal to 12–38 kg nitrogen ha^{-1} of fertilizer. This nitrogen-fixing inoculant mix added to the nitrogen fertilizer, mostly early, for small, but significant, increases in maize plant growth and yield.

1 | INTRODUCTION

Efficient fertilizer-nitrogen (N) management to meet maize (*Zea mays* L.) N needs is a challenge (Fernández et al., 2009; Ruffo et al., 2015) that is complicated by much fluctuation in soil N with different environmental and cultural conditions (Below, 2002). Additionally, the high energy cost and greenhouse gas emissions from fertilizer-N production and use, efficient cycling of soil-N, and the potential for N losses are concerns when striving to provide adequate N. Intrinsic non-fertilizer-N sources, such as mineralization of soil organic N, are important but insufficient to meet maize uptake demand, especially during the late vegetative and early reproductive growth stages (Bender et al., 2013), creating a need for fertilizer-N application for maximal productivity (Scharf, 2015). This variability in soil supply and plant use makes N management an inexact practice and highlights the need for new ways to enhance the availability of N during key plant growth periods.

In most agricultural lands, microbial diazotrophs, such as *Frankia* and *Azospirillum spp*., fix some atmospheric N to ammonium in free-living or in nonsymbiotic associations with maize and some other crops, but the contribution tends to be small relative to crop need (Baldani & Baldani, 2005; Bruijn, 2014; Olivares et al., 2013; Santi et al., 2013), and they have a high energy requirement (about 16 molecules of adenosine triphosphate per molecule N_2 reduced) that must be met from soil organic materials or root exudates (Marschner, 2011). Soil inoculation with diazotrophs has had limited success in enhancing N accumulation and cereal yield, likely due to abundant soil inorganic N following fertilizer-N application, which inhibits biological N fixation (Bloch et al., 2020; Martinez-Argudo et al., 2004). Gene-editing, however, has been used to promote N fixation in the presence of high available-N levels by altering the expression of the Nif genes responsible for the production and activity of the nitrogenase enzyme in *Azotobacter chroococcum* (Bageshwar et al., 2017), *Azotobacter vinelandii* (Ambrosio et al., 2017), and *Klebsiella variicola* plus *Kosakonia sacchari* (Wen et al., 2021).

One way to assess the effectiveness of an inoculation for fixation of atmospheric N is through decreases in δ^{15}N abundance in the soil (and respective plant uptake) relative to ^{14}N (Bedard-Haughn et al., 2003; Piccolo et al., 1996), because most agricultural soils have relatively high and homogeneous natural δ^{15}N abundance compared to other land uses (Bergersen et al., 1989; Ledgard & Peoples, 1988; Rerkasem et al., 1988).

A mixture of edited *K. variicola* plus *K. sacchari* has been commercialized and sold as PROVEN 40 (Pivot Bio) with claims that biologically-fixed N can potentially replace the equivalent of up to 45 kg ha^{-1} of fertilizer N, although there is a lack of peer-reviewed published data to support this claim or for estimation of the magnitude of N replacement values and when in the growth cycle that additional N is accumulated. The objective of this research was to determine if soil inoculation with an edited free-living nitrogen-fixing bacterial inoculant (NFI) can provide additional N to maize via biological N fixation and if the additional N supplied increases maize N uptake and productivity. We hypothesized that NFI activity would supply additional N to improve maize productivity at insufficient fertilizer-N rates and would be detected by a decrease in the proportion of δ^{15}N in both the soil and plant. We anticipated that maize productivity would improve with inoculation at fertilizer-N rates of 0–135 kg ha^{-1} and that there would be no benefit from inoculation at an N rate of 225 kg ha^{-1}, which is expected to be sufficient for maize yield goals of the region.

2 | MATERIALS AND METHODS

2.1 | Site descriptions

During the years 2019, 2020, and 2021, three maize field trials were established at Champaign, IL (CU; 40˚03′26″ N, 88˚14′15″ W). All CU sites were in different fields but within a 1.5 km radius. The soils in 2019 and 2021 were a Drummer silty clay loam (a fine-silty, mixed, superactive, mesic Typic Endoaquoll), and in 2020, an Elburn silt loam (a fine-silty, mixed, superactive, mesic Aquic Argiudoll) (USDA-NRCS, 2019). Planting was on June 2, 2019, May 9, 2020, and April 27, 2021. A fourth trial was planted on April 23, 2021, near Nashville, IL (NL; 38˚19′07″ N, 89˚20′17″ W). The soil was a Hoyleton silt loam (a fine, smectitic, mesic Aquollic Hapludalf). All trials had a 0%–2% slope; soybean was the previous crop, and tillage practices consisted of a deep-ripping chisel plow in the fall followed by a field cultivator in the spring before planting. Composite soil samples of eight cores from the 0- to 30-cm depth were collected for each trial before planting and analyzed using the Mehlich III extraction with inductively coupled plasma optical emission spectroscopy quantification (ICP OES) (Mehlich, 1984) at A&L Great Lakes Laboratories, Inc. (Table 1).

Temperatures for all four site-years (SY) were similar to the respective 30-year averages, and seasonal precipitation was within 15% of the 30-year average at each SY, with some variation in distribution (Supplemental Table S1). Therefore, CU in 2019 and CU in 2020 had relatively wet springs and dry summers in contrast to CU and NL 2021, which had dry springs with wet summers.

Core Ideas

- N-fixing inoculant supplements maize with atmospheric N_2 during vegetative growth.
- Inoculant-supplied N increases are a function of plant biomass, not tissue concentration.
- Biomass response to inoculation is greatest at V8 (+4.8%) but diminishes by maturity.
- Averaged over N rates, grain yield was increased by 1.2% when inoculated.

2.2 | Agronomic practices and treatment descriptions

Maize plots were established with a precision plot planter (SeedPro 360; ALMACO) at a density of 89,000 plant ha^{-1} at CU or 84,000 plant ha^{-1} at NL. Maize hybrid G12W66 (Syngenta) was used in 2019 and 2020, while DKC64-34 (Bayer Crop Sciences) was used in 2021. At planting, tefluthrin ([2,3,5,6-tetrafluoro-4-methylphenyl]methyl-[1α,3α]-[Z]-[±] -3-[2-chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylate) was applied as Force 6.5G (Syngenta) in furrow at an a.i. rate of 65 g per 300 m of crop row for control of seedling insect pests. Weed control consisted of a preemergence application of Acuron (Syngenta) at a product rate of 5.8 L ha^{-1} to provide S-metolachlor (2-chloro-*N*-[2-ethyl-6-methylphenyl]-*N*-[2-methoxy-1-methylethyl]acetamide), atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine), mesotrione (2-[4-[methylsulfonyl]-2-nitrobenzoyl]cyclohexane-1,3-dione), and bicyclopyrone (bicyclo[3.2.1]october-3-en-2-one, 4-hydroxy-3-[[2-[methoxyethoxy)methyl]-6-[trifluoromethyl]-3-pyridinyl]carbonyl]) with Infantry 4L (Growmark) at a product rate of 1.46 L ha^{-1} to supply additional atrazine. A postemergence application of glyphosate [*N*-(phosphonomethyl)glycine], atrazine, dicamba (3,6-dichloro-o-anisic acid), and tembotrione (2-[2-chloro-4-methylsulfonyl]-3-[(2,2,2-trifluoroethoxy)methyl]benzoyl]-1,3-cyclohexanedione) were supplied as Roundp PowerMAX 3 (Bayer), Infantry 4L, and DiFlexx Duo (Bayer) at product rates of 2.3, 2.92, and 0.44 L ha^{-1}, respectively.

An NFI containing gene-edited *K. variicola* (1 × 10^8 colony forming units [CFU] mL^{-1}) (PROVEN; Pivot Bio) was applied in furrow at planting at a product rate of either 4.9 L ha^{-1} (2019) or 0.9 L ha^{-1} (2020) with a planter-attached liquid in-furrow system (Surefire Ag Systems) (Figure S1). In 2021, gene-edited *K.* variicola (1 × 10^8 CFU mL^{-1}) with gene-edited *K. sacchari* (1 × 10^8 CFU mL^{-1}) (PROVEN 40; Pivot Bio) was applied in furrow at planting at a product rate of 0.9 L ha^{-1}. The rates and microbial blends of applied NFI differed in the 3 years due to different product formulations, and all applications were supplied based on the product-labeled rates respective to formulation available in each year. The inoculant was blended with water for a total application volume of either 74.8 L ha^{-1} (2019) or 112 L ha^{-1} (2020 and 2021). Treatments were a complete factorial with or without NFI, each applied with urea-N (46-0-0) preplant broadcast and incorporated at 0, 45, 90, 135, or 225 kg N ha^{-1}, with 135 N ha^{-1} or less considered to be yield-limiting. The randomized complete block design had eight replications in 2019 and 2020 and six replications in 2021. Experimental units (60 or 80 per SY) were four rows wide with 0.76-m row spacing by 11.4 m in length with a 0.76-m alley between each range of plots.

TABLE 1 Preplant soil test values of four sites used in the evaluation of the effect of in-furrow applications of a nitrogen-fixing bacterial inoculant on maize production at Champaign (CU), IL, in 2019, 2020, and 2021 and at Nashville (NL), IL, in 2021.

Site-year	OM (mg kg^{-1})	CEC (cmol(+) kg^{-1})	pH	NO_3-N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	S (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})
CU 2019	33	26.6	6.4	4.7	3.8	34	127	3515	610	9	1.2	0.4
CU 2020	37	18.5	6.1	4.1	4.6	18	102	2298	382	10	1.0	0.2
CU 2021	35	18.2	6.7	10.2	6.5	15	103	2430	546	6	1.1	0.6
NL 2021	14	7.5	5.9	5.9	4.9	20	58	1065	98	6	0.9	0.3

Note: Soils were sampled preplant at the 0- to 30-cm depth and analyzed with Mehlich III extraction.
Abbreviations: CEC, cation exchange capacity; OM, organic matter.

2.3 | Measured variables

At both the V8 (eight-fully collared leaves) and R1 (silk emergence) growth stages, four random, but representative, plants were sampled at the soil surface from plot rows 1 and 4 to determine plant shoot biomass, N accumulation, and partitioning at all trial sites. Plants were separated into leaf, stalk, and reproductive (only at the R1 growth stage) tissue components. Reproductive tissues included the ear shoots, husks, and tassel. After dividing the plants into their respective tissue components, they were dried to constant weight in a forced air oven at 75°C to determine the dry weight, and the dried plant partitions were ground to pass through a 2-mm mesh screen (Thomas-Wiley Laboratory Mill Model 4; Thomas Scientific). Subsamples were evaluated for N concentration using a combustion-based analyzer (EA1112; CE Elantech). The N concentration was multiplied by the dry plant partition biomass and planting density to determine accumulated N in each plant fraction. A hailstorm caused a uniform ∼30% leaf defoliation at 10 days before flowering for CU 2020, so the R1 vegetative plant tissue was not fractioned, and determinations were for total vegetative, reproductive, and total biomass and N concentration.

Leaf subsamples collected at V8 during the 2020 and 2021 field trials (three of the four SY), V8 stalk samples collected during 2021 (two of the four SY), and R1 leaf, stalk, and reproductive tissue subsamples collected in 2021 (two of the four SY) were analyzed for δ^{15}N isotope abundance at Isotope Tracer Technologies. In 2021, four soil cores (0- to 30-cm depth) from the NL location were collected at the V8 growth stage from the seed furrow of each plot, homogenized for a per-plot composite, and shipped to Isotope Tracer Technologies without drying or refrigeration for δ^{15}N abundance determination.

Maize grain yield and harvest moisture were measured by harvesting the center two rows of each plot with a research plot combine (R1 Rotary Single Plot Combine; ALMACO), and the subsequent grain yield values were standardized to 0 g kg^{-1} moisture. Kernel weight was determined from 300 kernel subsamples of the harvested grain and adjusted to 0 g kg^{-1} moisture. Kernels per area were calculated by dividing the total plot dry grain weight by the average kernel weight and dividing by plot area.

2.4 | Statistical analysis

Measured variables were evaluated using a mixed model analysis of variance with PROC MIXED of SAS (version 9.4; SAS Institute), and mean separation was determined with the Fisher's LSD using the PDIFF option of the LSMEANS statement. NFI treatment and fertilizer-N rate were considered as fixed effects, with SY and replication nested within SY as random factors in the model. The least significant differences between treatments were declared at $p < 0.05$. Homogeneity of variance was checked with the Brown–Forsythe modification of the Levene test in PROC GLM of SAS, and normality of residuals was assessed with the Shapiro–Wilk test using PROC UNIVERIATE of SAS. Outlier analysis was conducted using influence diagnostics and Pearson residuals.

To determine the relationships between N Rate and V8 N content, R1 N content, or grain yield, quadratic regression models were fit using R statistical software (version 4.4.1) (R Core Team, 2023) and visualized with *ggplot2* (Wickham et al., 2016). Data were separated into two groups, no NFI or NFI, and a separate model was fit for each group. Fertilizer-N equivalents of NFI were then calculated by using the predictions of the model to estimate the N rate at which NFI produced the same level of the dependent variable with no NFI at specified N rates of 45, 90, 135, or 225 kg fertilizer N ha^{-1}.

3 | RESULTS

3.1 | Plant biomass and N accumulation

The random interaction of the NFI × SY was only significant ($p < 0.10$) for V8 stalk N content, V8 total N content, R1 leaf δ^{15}N, and R1 reproductive δ^{15}N. The random three-way interaction of NFI × N rate × SY was only significant ($p < 0.10$) for V8 leaf N content (Table S2). Therefore, only the combined SY analyses are displayed in the main text, with fixed effect p-values presented in Table 2.

Of the 21 measured variables, N rate was significant for 19, NFI for 12, and the N rate × NFI treatment interaction only for two variables (Table 2). All V8 biomass components increased with fertilizer-N additions up to the 90 kg N ha^{-1} rate, and averaged across N rates, there was 45 kg ha^{-1} more total dry plant biomass with NFI compared with no NFI (Figure 1). Plant N accumulation at V8 increased with fertilizer-N additions up to the 135 kg N ha^{-1} rate (Figure 2), and there was an additional 2.1 kg N ha^{-1} (+4.8%) uptake with NFI (Figure S2). NFI-related plant N accumulation responses were the result of differences in plant biomass (Figure 1), as opposed to N concentration, as there were no effects of NFI on plant tissue N concentrations (data not shown).

Nitrogen rate significantly increased the vegetative, reproductive (ear shoot and tassel), and total biomass and N accumulation at R1 (Figures 3 and 4; Figure S3). Averaged across N rates, NFI resulted in greater total ear shoot and tassel biomass (Figure 3) and greater total N accumulation (Figure 4) at R1, suggesting that the NFI may be increasing ear size or promoting earlier ear shoot development. Similar to the V8 growth stage, increases in R1 plant N accumulation were the result of greater plant biomass (Figure 3) as opposed to plant N concentration (data not shown).

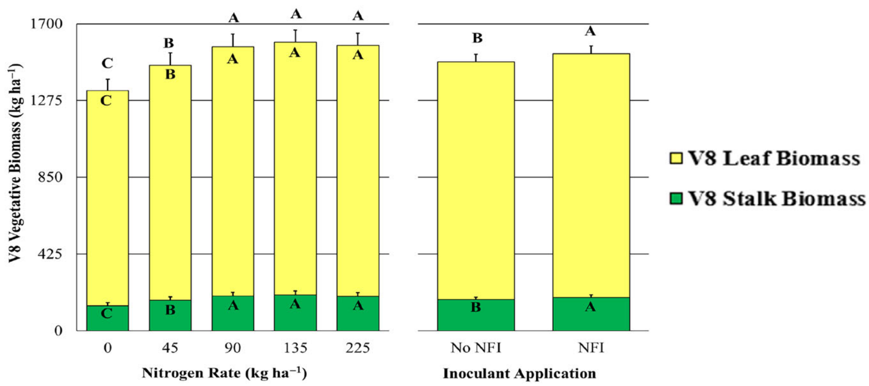

FIGURE 1 Influence of fertilizer-N rate and nitrogen-fixing bacterial inoculant (NFI) treatment on V8 leaf, stalk, and total maize plant biomass accumulation averaged across four trials in Illinois in 2019, 2020, and 2021. Different letters indicate significant differences between treatment levels at the $p < 0.05$ significance level. Letters within bars are specific to that plant fraction, while letters above the bars refer to differences in total biomass accumulation. No letters indicate a nonsignificant effect. Handles represent the standard error of the mean.

TABLE 2 Tests of fixed effects on maize biomass and nitrogen accumulation, soil δ^{15}N, grain yield, and yield components as influenced by five levels of nitrogen (N) rate, two levels of nitrogen-fixing bacterial inoculant (NFI), and their interaction across four trials conducted in Illinois during 2019–2021.

Measured variable	Fixed sources of variation		
	N Rate	NFI	N Rate × NFI
	$p > F$		
V8 leaf biomass	<0.0001	0.0723	0.5539
V8 stalk biomass	<0.0001	0.0174	0.3579
V8 total biomass	<0.0001	0.0499	0.4839
V8 leaf N content	<0.0001	0.0098	0.7320
V8 stalk N content	<0.0001	0.0226	0.6340
V8 total N content	<0.0001	0.0056	0.6381
R1 leaf and stalk biomass	<0.0001	0.0989	0.2113
R1 ear shoot and tassel biomass	<0.0001	0.0357	0.0444
R1 total biomass	<0.0001	0.0580	0.0896
R1 leaf and stalk N content	<0.0001	0.0684	0.2619
R1 ear shoot and tassel N content	<0.0001	0.0978	0.2774
R1 total N content	<0.0001	0.0150	0.1540
V8 soil δ^{15}N[a]	0.4555	0.0141	0.0283
V8 leaf δ^{15}N[b]	<0.0001	0.0357	0.4264
V8 stalk δ^{15}N[c]	0.0176	0.0929	0.7465
R1 leaf δ^{15}N[c]	0.1698	0.0315	0.7071
R1 stalk δ^{15}N[c]	<0.0001	0.8821	0.2644
R1 ear shoot and tassel δ^{15}N[c]	0.0251	0.0038	0.0504
Grain yield	<0.0001	0.0779	0.7310
Kernel number	<0.0001	0.0416	0.3389
Kernel weight	<0.0001	0.4277	0.5820

Note: Each trial contained six or eight replications, resulting in $n = 56$ for main effect of nitrogen rate, $n = 140$ for main effect of NFI, and $n = 28$ for their interaction when analyzed across site-years.

Abbreviations: R1, silking stage; V8, 8-leaf stage.

[a,b,c]Data only collected and analyzed for one trial[a], two trials[b], or three trials[c].

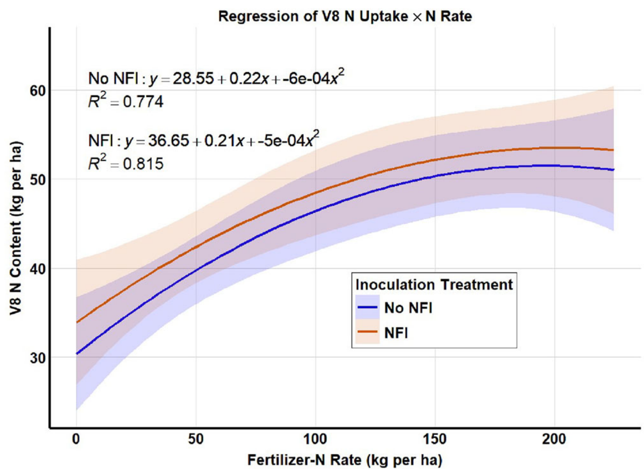

FIGURE 2 Quadratic regression of fertilizer-N rate and nitrogen-fixing bacterial inoculant (NFI) treatment on V8 total maize plant nitrogen content analyzed across four site-years in Illinois in 2019, 2020, and 2021. Shaded boundaries represent the 95% confidence interval for each respective level of inoculation.

3.2 | δ^{15}N abundances

Soil δ^{15}N abundance was unchanged by N rate but was reduced with NFI application (Table 3), and there was a significant interaction of N rate × NFI where soil δ^{15}N was reduced by the NFI at the 90 and 225 kg N ha^{-1} rates but not for the remaining N rates. NFI application decreased leaf and stalk δ^{15}N abundance when averaged across N rates at the V8 growth stage (Table 3), and similarly resulted in lower overall levels of leaf and reproductive tissue δ^{15}N abundance at R1 (Table 4).

3.3 | Grain yield and yield components

The main effects of NFI treatment and N rate were significant sources of variation on grain yield and kernel number, while kernel weight was only affected by N rate (Table 5). Each incremental increase in the fertilizer rate resulted in greater

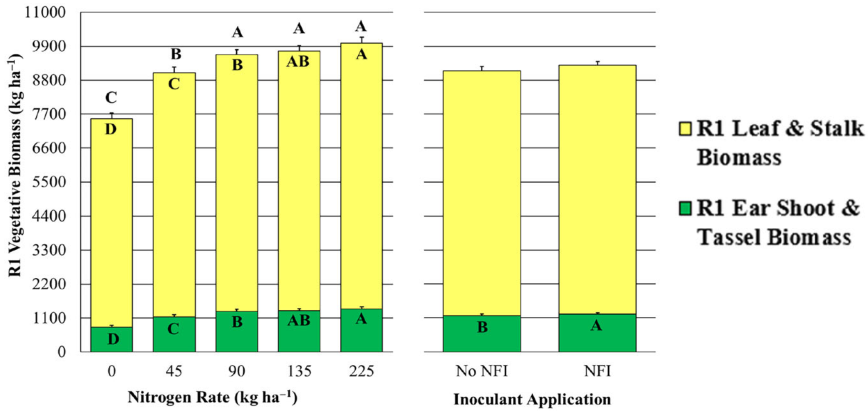

FIGURE 3 Influence of fertilizer-N rate and nitrogen-fixing bacterial inoculant (NFI) treatment on R1 vegetative (leaf and stalk), reproductive (ear shoot and tassel), and total maize plant biomass accumulation analyzed across four site-years in Illinois in 2019, 2020, and 2021. Different letters indicate significant differences between treatment levels at the $p < 0.05$ significance level. Letters within bars are specific to that plant fraction, while letters above the bars refer to differences in total biomass accumulation. No letters indicate a nonsignificant effect. Handles represent the standard error of the mean.

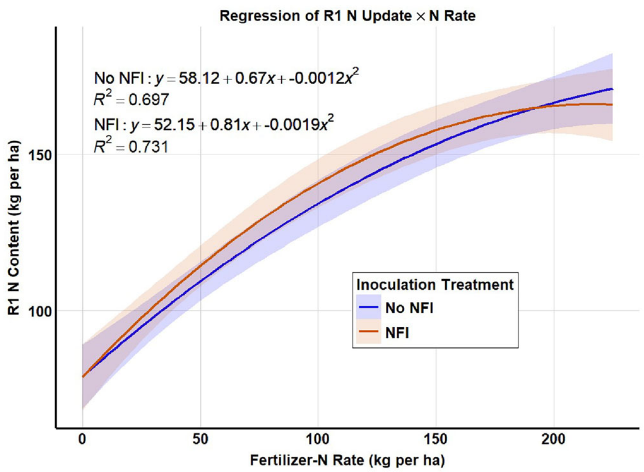

FIGURE 4 Quadratic regression of fertilizer-N rate and nitrogen-fixing bacterial inoculant (NFI) treatment on R1 total maize plant nitrogen content analyzed across four site-years in Illinois in 2019, 2020, and 2021. Shaded boundaries represent the 95% confidence interval for each respective level of inoculation.

grain yield, kernel number, and kernel weight, and when averaged across the N rates, applications of NFI increased grain yield by 0.11 Mg ha^{-1}, which corresponded to significant increases in kernel number (Table 5). Individual SY yield, kernel number, and kernel weight changes due to fertilizer-N rates and NFI provision status are presented in Tables S3–S6.

3.4 | Fertilizer-N replacement value

Using quadratic regression models for selected dependent variables as a function of fertilizer-N rate, the fertilizer-N equivalents of NFI were, on average, 38.5, 12.1, and 11.5 kg N ha^{-1} to achieve the same level of V8 N content, R1 N content, and grain yield, respectively, with no NFI (Table 6). Excluding the 225 kg ha^{-1} fertilizer-N rate, which was shown to be non-limiting (i.e., no additional increase in biomass or yield when compared to 135 kg ha^{-1} fertilizer-N rate), and averaging the NFI-induced fertilizer-N equivalents at the moderate N rates (90 or 135 kg fertilizer N ha^{-1}) revealed NFI-induced fertilizer-N equivalents of 21.4, 14.7, and 15.9 kg N ha^{-1} for V8 N content, R1 N content, and grain yield, respectively (Table 6; Figure 5).

4 | DISCUSSION

Applications of an NFI led to increases in plant N accumulation at the V8 and R1 growth stages as a result of greater plant biomass accumulations without changing tissue N concentrations (Figures 1–4; Figures S2 and S3). Similar results have been observed previously with other NFI applications (Tang et al., 2020) and result from a "dilution effect," where the relative rate of dry matter accumulation is proportional to the rate of nutrient accumulation (Jarrell & Beverly, 1981). These findings show that NFI are supplementing greater availability of N in the rooting zone during the growth period when the crop has exponential N uptake (Bender et al., 2013) and when mineralization of organic N is too slow to accommodate this N demand (Below, 2002; Scharf, 2015). Furthermore, the consistent increases across all levels of N rates indicate that the NFI was not hindered by the application of fertilizer N, which is often observed in legume and symbiotic N-fixation systems. Grain yield increases induced by NFI application in this and in previous studies (Dobbelaere et al., 2001; Gholami et al., 2009) were the result of greater kernel production, confirming that additional N was available during the mid- to late-vegetative stages, when maize kernel row number and kernel length are determined (Abendroth et al., 2011). The NFI decreased the relative δ^{15}N abundance in the soil for the 2021 NV site and in the V8 and R1 plant samples measured across multiple SY (Tables 3 and 4), showing that the increases in plant biomass and N accumulation were aided by biologically-fixed atmospheric N from the NFI. Additionally, NFI treatment led to increases in R1 reproductive tissue biomass (Figure 3), which has been reported to induce both earlier flowering and longer

TABLE 3 Influence of fertilizer-N rate, nitrogen-fixing bacterial inoculant (NFI), and their interaction on V8 soil $\delta^{15}N$ abundance at Nashville, IL, in 2021, V8 stalk maize plant $\delta^{15}N$ abundance averaged across two trials at Champaign and Nashville, IL, in 2021, and V8 leaf maize plant $\delta^{15}N$ averaged across three trials at Champaign and Nashville, IL, in 2020 and 2021.

	V8 soil $\delta^{15}N$[a] ($\delta^{15}N$ (‰))		V8 leaf $\delta^{15}N$[b] ($\delta^{15}N$ (‰))		V8 stalk $\delta^{15}N$[c] ($\delta^{15}N$ (‰))	
	Nitrogen-fixing inoculant treatment					
Nitrogen rate (kg N ha^{-1})	No NFI	NFI	No NFI	NFI	No NFI	NFI
0	6.29	6.22	4.88	4.83	2.33	2.18
45	6.63	6.51	4.65	4.37	2.98	2.74
90	6.65	6.39*	4.28	3.80	1.95	2.03
135	6.57	6.73	3.70	3.74	2.17	1.85
225	6.63	6.40*	3.69	3.49	2.41	1.81
Means	6.55 B	6.45A	4.24B	4.05A	2.37	2.12

Note: Different letters within a measured variable indicate significant differences for the main effect of NFI at $p < 0.05$.
Abbreviation: V8, 8-leaf stage.
[a]N rate LSD ($p < 0.05$) for V8 soil $\delta^{15}N$ = NS.
[b]N rate LSD ($p < 0.05$) for V8 leaf $\delta^{15}N$ = 0.30.
[c]N rate LSD ($p < 0.05$) for V8 stalk $\delta^{15}N$ = 0.56.
*NFI is significantly different from No NFI within respective N rate (N Rate × NFI; $p = 0.0283$).

TABLE 4 Influence of fertilizer-N rate, nitrogen-fixing bacterial inoculant (NFI), and their interaction on R1 leaf, stalk, and reproductive maize plant $\delta^{15}N$ abundance averaged across two trials in Illinois in 2021.

	R1 leaf $\delta^{15}N$[a] ($\delta^{15}N$ (‰))		R1 stalk $\delta^{15}N$[b] ($\delta^{15}N$ (‰))		R1 ear shoot and tassel $\delta^{15}N$[c] ($\delta^{15}N$ (‰))	
	Nitrogen-fixing inoculant treatment					
Nitrogen rate (kg N ha^{-1})	No NFI	NFI	No NFI	NFI	No NFI	NFI
0	3.60	3.49	1.99	1.65	4.66	4.20
45	3.94	3.85	2.47	2.94	5.41	5.54
90	3.66	3.40	3.20	3.07	5.27	5.24
135	3.69	3.67	3.56	3.74	5.29	4.92
225	4.19	3.90	4.43	4.35	5.28	4.54
Means	3.82B	3.66A	3.13	3.15	5.18B	4.89A

Note: Different letters within a measured variable indicate significant differences for the main effect of NFI at $p < 0.05$.
Abbreviation: R1, silking stage.
[a]N rate LSD ($p < 0.05$) for R1 leaf $\delta^{15}N$ = 0.49.
[b]N rate LSD ($p < 0.05$) for R1 stalk $\delta^{15}N$ = 0.57.
[c]N rate LSD ($p < 0.05$) for R1 ear shoot and tassel $\delta^{15}N$ = 0.64.

TABLE 5 Influence of fertilizer-N rate, nitrogen-fixing bacterial inoculant (NFI), and their interaction on grain yield, kernel number, and average kernel weight of maize averaged across four site-years in Illinois in 2019, 2020, and 2021.

	Grain yield[a] (Mg ha^{-1})		Kernel number[b] (kernels m^{-2})		Kernel weight[c] (mg kernel^{-1})	
	Nitrogen-fixing inoculant treatment					
Nitrogen rate (kg N ha^{-1})	No NFI	NFI	No NFI	NFI	No NFI	NFI
0	6.68	6.69	3172	3163	208	211
45	8.13	8.28	3641	3779	221	218
90	9.35	9.59	4007	4134	231	231
135	10.71	10.86	4429	4480	239	242
225	11.68	11.69	4711	4707	249	253
Means	9.31B	9.42A	3992B	4053A	230	231

Note: Different letters within a measured variable indicate significant differences for the main effect of NFI at $p < 0.05$.
[a]N rate LSD ($p < 0.05$): for grain yield = 0.28.
[b]N rate LSD ($p < 0.05$) for Kernel number = 104.
[c]N rate LSD ($p < 0.05$) for Kernel weight = 4.

TABLE 6 Fertilizer-N equivalent of a nitrogen-fixing bacterial inoculant (NFI) compared to no NFI when assessing maize V8 N accumulation, R1 N accumulation, and grain yield variables at varying fertilizer-N rates.

N-rate (kg ha^{-1})	V8 N accumulation	R1 N accumulation	Grain yield
	Fertilizer-N equivalent of NFI		
	kg ha^{-1} (N-Rate required to match)		
45	17.0 (28.0)	7.0 (38.0)	11.7 (33.3)
90	18.3 (71.7)	13.0 (77.0)	14.8 (75.2)
135	24.5 (110.5)	16.4 (118.6)	17.0 (118.0)
225	94.3 (130.7)	n.e.[a]	2.5 (222.5)
Δ Means	38.5	12.1	11.5

Note: Values in parenthesis represent the amount of fertilizer N required with NFI to achieve the same level of dependent variable as the respective N-rate with no NFI. Fertilizer-N equivalents were determined by identifying the value of the dependent variable with no NFI at the specified N rates of 45, 90, 135, or 225 kg N ha^{-1} and then comparing those to the respective NFI regression model to assess at what N rate the NFI was able to achieve the same level of the dependent variable. Abbreviations: R1, silking stage; V8, 8-leaf stage.

[a]Not estimable as the R1 N accumulation value from no NFI at 225 kg N ha^{-1} was above the regression curve for NFI and therefore fertilizer-N equivalent could not be predicted.

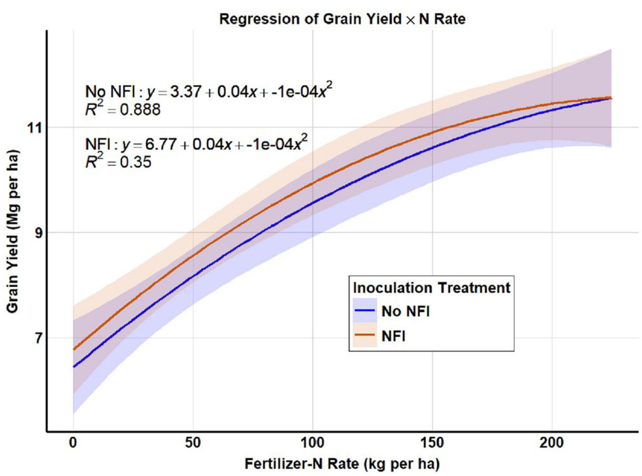

FIGURE 5 Quadratic regression of fertilizer-N rate and nitrogen-fixing bacterial inoculant (NFI) treatment on maize grain yield analyzed across four site-years in Illinois in 2019, 2020, and 2021. Shaded boundaries represent the 95% confidence interval for each respective level of inoculation.

ear lengths (Baral & Adhikari, 2013; Victor et al., 2019), and which are well-known phenomena associated with an enhanced or an improved supply of N to maize (Ahmad et al., 2018; Ciampitti & Vyn, 2011; Ta & Weiland, 1992).

While the NFI resulted in consistent statistically significant improvements in biomass and N accumulation, the overall effects were modest, +2.1 (4.8%) and +4.6 (3.7%) kg N ha^{-1} at V8 and R1, respectively, and +0.11 (1.2%) Mg ha^{-1} for grain yield, and the ability to statistically detect small differences was likely due to the high degree of replication (six to eight replications) across four SY. The decreasing magnitudes of NFI-induced increases also show that the NFI effect is greater in the early-season during the vegetative growth stages and diminishes by physiological maturity, indicating either a decrease in N-fixation capability of the NFI as the season progresses or that soil N mineralization rates are able to overcome any early-season N deficits. Collectively, the increases in vegetative N and the changes in δ^{15}N show that inoculation of NFI to maize can serve as a third source of N by providing additional N from the atmosphere from biological N fixation.

Despite the importance of N to maize yield, previous results of different NFI treatments to maize productivity have been highly variable, with reports of no effects or slight yield decreases (D. Franzen, Camberato, et al., 2023; Freitas & Stamford, 2002; Kifle & Laing, 2016), tendencies to increase yields (Dobbelaere et al., 2001; Davis, 2021; Kifle & Laing, 2016), or in some cases, reports of statistically significant increases in yield (Dobbelaere et al., 2001; Gholami et al., 2009; Okon & Vanderleyden, 1997). These inconsistencies are not surprising, as NFI are a living input that must adapt to the environment in which they are supplied just as plants adapt season-to-season based on weather conditions. D. W. Franzen, Wick et al. (2023) identified that dry periods or prolonged wet conditions can result in reduced levels of asymbiotic N fixation and that appropriate temperature and sufficient rainfall benefit microbial activity. The four SY in this study were all within 15% of average precipitation for their respective regions with uniform temperatures, resulting in consistent responses of NFI on grain yield, with an average effect of +0.11 Mg ha^{-1} (Table 5). However, this yield enhancement, while significant at $p < 0.05$, is small when put in the context of on-farm expectations from an additional input and the need for a positive return on investment. With the costs of different NFI products marketed at ranges from \$25 to \$75 ha^{-1}, and assuming an average urea-N price of \$1.19 kg^{-1} (Schnitkey, 2016) and a grain value of \$4 per 21.5 kg of grain, it would require a 0.13–0.40 Mg ha^{-1} yield increase to profit with the use of an NFI. Using these price estimates and the fertilizer-N values from the regression models, the \$ value of the fertilizer-N equivalent supplemented by NFI at V8 was \$46 ha^{-1}, and at R1 and harvest was \$14 ha^{-1}, all of which are below the average cost of typical NFI products, and therefore in today's market the \$ per unit value of N from NFI is higher than the cost for fertilizer N. These cost estimates, however, do not take into account the ongoing price fluctuations of grain and fertilizer N, the environmental and economic costs associated with fertilizer-N production and transport, the current need to reduce N losses from off-target fertilizer N, or the potential for future governmental regulation of N fertilizers or sustainability incentives of reducing fertilizer-N inputs. Moreover, if NFI was used to reduce total N inputs by the 11.5 kg N ha^{-1} fertilizer-N value shown here across a majority of maize production hectares, it could lead to notable environmental benefits.

5 | CONCLUSION

Increases in N accumulation in all plant fractions were observed across the growing season in response to the NFI application, and δ^{15}N abundance measurements confirmed that some of the additional N accumulation was derived from biological N fixation beyond the native levels of asymbiotic N fixation already occurring in the soil system. NFI-driven increases in plant N accumulation were associated with modest, but statistically significant, increases in grain yield as a function of increased kernel production. Given the current prices for NFI, fertilizer N, and maize grain, the NFI effects in this study were not large enough to warrant a significant replacement of fertilizer N but did identify NFI as a third source of N that can supplement soil and fertilizer supply. As such, NFI may be an option in future precision N management systems to aid in providing maize plants with adequate levels of N in areas of the fields where fertilizer N was lost or supplementing N to field areas where applied fertilizer rates were still yield-limiting. This work reveals the potential of using an NFI to increase the N use of maize but shows that the season-long benefit of fixed-N is yet to be fully optimized in maize production and that further work is needed if NFI are to serve as a reliable source of N in maize production.

AUTHOR CONTRIBUTIONS

Logan P. Woodward: Data curation; formal analysis; investigation; methodology; software; writing—original draft; writing—review and editing. **Connor N. Sible**: Data curation; investigation; methodology; supervision; validation; writing—review and editing. **Juliann R. Seebauer**: Project administration; resources; supervision; writing—review and editing. **Frederick E. Below**: Funding acquisition; project administration; resources; supervision; writing—original draft; writing—review and editing.

ACKNOWLEDGMENTS

This work is supported in part by the USDA National Institute of Food and Agriculture, Multistate Hatch project NC1200, and IL-Agricultural Experiment Station projects ILLU-802-904 and ILLU-802-990 at the College of Agricultural, Consumer, and Environmental Sciences, University of Illinois at Urbana-Champaign. The authors especially appreciate the cooperation and generosity of Bartling Farms at Nashville, IL.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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How to cite this article: Woodward, L. P., Sible, C. N., Seebauer, J. R., & Below, F. E. (2025). Soil inoculation with nitrogen-fixing bacteria to supplement maize fertilizer need. *Agronomy Journal*, e21729. https://doi.org/10.1002/agj2.21729