Effect of root-associated bacteria on corn nitrogen use efficiency

by

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Abstract

Nitrogen (N) is a critical input for corn (*Zea mays* L.) production, essential for optimizing crop growth and yield. However, a significant portion of synthetic N fertilizers is lost to the environment, leading to negative ecological impacts, such as groundwater contamination and greenhouse gas emissions. Optimizing nitrogen management is, therefore, crucial for sustainable agricultural production. This study evaluated the bacterial inoculant Proven®, which colonizes corn roots with nitrogen-fixing bacteria, potentially enhancing nitrogen use efficiency (NUE), biomass accumulation, and grain yield. The experiment employed a split-plot randomized complete block design for over three years (2021-2023) at the Agronomy North Farm, Manhattan, KS. The main plot treatments consisted of four nitrogen rates (0, 56, 112, and 168 kg N ha^{-1}) applied as urea, while Proven®, applied in-furrow at planting, served as the subplot treatment, with six replicates. Plant biomass and nitrogen uptake were measured at the vegetative eight-leaf (V8), tasseling (VT), and maturity (R6) growth stages, with grain yield and NUE metrics calculated at R6. Proven® demonstrated a stage-specific effect on dry matter accumulation and nitrogen uptake, particularly at the V8 and VT stages, when treated plants showed increased dry matter and enhanced nitrogen uptake compared to untreated plants. Despite these improvements, the overall effect on grain yield and NUE indices was limited, with no significant differences observed across the three years. Proven®-treated plants exhibited greater nitrogen uptake at earlier growth stages, but this did not consistently translate into improved NUE metrics. While the production metrics were not significantly enhanced, the use of Proven® offers potential environmental benefits by facilitating biological nitrogen fixation. This could reduce reliance on synthetic fertilizers, decrease nitrogen losses, and mitigate environmental issues such as groundwater contamination and greenhouse gas emissions. These findings suggest that while Proven®'s effectiveness may vary

depending on environmental conditions and growth stages, its role in promoting sustainable agriculture and reducing the ecological footprint of nitrogen fertilization warrants further investigation, with attention to factors such as climate and soil variables to optimize its application.

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Dedication

I dedicate this achievement to my family, especially to my wife, my father, my mother, my sister, and our soon-to-arrive baby. Their unwavering love and support have been fundamental in every step of this journey. They stood by me during both the happy and challenging moments, reminding me never to give up. I could not have accomplished this without their belief in me. I also extend my heartfelt thanks to my wife's family, whose encouragement and support played an equally important role in this journey. Thank you all for being there for me.

Chapter 1 - Literature Review

Sustainable Nitrogen Fixation in Corn: Exploring the Role of Asymbiotic Root-Associated Diazotrophic Bacteria

The availability of nitrogen (N) is vital for the growth and development of cereal crops in the agricultural sector. Maize (*Zea mays L.*), a major cereal crop worldwide, requires significant amounts of nitrogen during its development for key processes like photosynthesis and synthesis of amino acids, proteins, and enzymes (Hirel et al., 2005). Traditionally, nitrogen fertilizers derived from the energy-intensive Haber-Bosch process have been relied upon to meet the nitrogen demands of crops like corn, rice, and wheat (Ladha et al., 2016). Indeed, the productivity of cereals, including wheat, rice, and maize, has closely followed the increase in fertilizer consumption, especially nitrogen, phosphate, and potassium, highlighting a strong relationship between fertilizer use and cereal crop production (Curatti & Rubio, 2014).

It is essential to note that 45% of the world's dietary energy for cereal crops comes from plant-available nitrogen, emphasizing the crucial need to meet this demand for global food security (Griffiths, 2014). With the global population continuously increasing (UN DESA, 2021), there is a pressing requirement to increase agricultural production by 60 to 70% before 2050 (Bruinsma, 2009; FAO, 2015). This increase is necessary to feed the growing population sustainably and will require an additional 1 billion Mg of cereal production (FAO, 2015). Consequently, the role of nitrogen becomes even more critical in this context (Erisman et al., 2008). However, the widespread use of synthetic nitrogen fertilizers has led to significant environmental repercussions, including groundwater contamination and escalated greenhouse gas emissions (IPCC, 2014).

There has been a growing interest in sustainable alternatives to synthetic nitrogen fertilizers to address these challenges. One alternative is the Biological Nitrogen Fixation (BNF), which

involves prokaryotes (diazotrophs) capable of converting atmospheric nitrogen (N_2) into ammonia (NH_3) through the action of the enzyme nitrogenase (Franche et al., 2008). This process is catalyzed by nitrogenases but is energetically costly, requiring 16 ATP per reduced N_2 molecule (Curatti & Rubio, 2014). Diazotrophs form various associations with host plants, with the plant providing carbon through root exudates to feed the microbes that fix atmospheric nitrogen for the plant's use, resulting in successful interaction (Zhu et al., 2002; Dardanelli et al., 2008; Dardanelli et al., 2010).

Unlike legumes (Fabaceae), which form specialized symbiotic relationships with diazotrophic rhizobia within root nodules, non-legume crops such as maize establish associations with diazotrophic bacteria that inhabit the rhizosphere, either on the root surface or inside the root tissue (de Bruijn, 2015). These root-associated nitrogen-fixing bacteria have the potential to supply significant amounts of nitrogen to cereal crops, thereby decreasing reliance on synthetic nitrogen fertilizers. Recent studies have assessed the strategies and challenges of leveraging diazotrophic bacteria for agricultural benefits in non-leguminous crops (Mus et al., 2016; Bloch, Ryu, et al., 2020; Bloch, Clark, et al., 2020).

This literature review explores the potential of asymbiotic nitrogen fixation by root-associated diazotrophic bacteria as a sustainable solution for improving nitrogen use efficiency and reducing the environmental impact that synthetic fertilizers can cause. The review will cover the environmental impacts of nitrogen fertilizers, the role of asymbiotic nitrogen fixation, genetic modifications for enhanced nitrogen fixation, and strategies to enhance nitrogen use efficiency in cereal crop production.

Environmental and Agronomic Challenges of Synthetic Nitrogen Fertilizers

Despite the essential role of nitrogen fertilizers in fulfilling crop demands, their production and usage contribute significantly to environmental degradation. Approximately 5% of global greenhouse gas (GHG) emissions are attributed to synthetic nitrogen fertilizer production and application (Gao & Cabrera Serrenho, 2023). The process of creating synthetic nitrogen is associated with environmental concerns, high production costs, and relies heavily on fossil fuels (Galloway et al., 2004; Fowler et al., 2013).

Additionally, the global application of nitrogen fertilizer results in the loss of half of the nitrogen due to leaching and as a nitrous oxide gas (Lassaletta et al., 2014). This inefficiency contributes to groundwater contamination and escalates GHG emissions. Synthetic nitrogen fertilizers, mainly ammonium (NH_4^+) or nitrate (NO_3^-), are crucial in agriculture but are prone to losses. Ammonium can volatilize into ammonia (NH_3) gas, especially when fertilizers are surface-applied without soil incorporation, leading to increased volatilization and reduced fertilizer efficiency (Pan et al., 2016).

Moreover, the susceptibility of NO_3^- to leaching or runoff, coupled with its conversion to gases such as nitric oxide (NO), nitrous oxide (N_2O), or dinitrogen (N_2) gas through denitrification under anaerobic conditions, further complicates the nitrogen cycle (Robertson & Groffman, 2024). These processes contribute significantly to groundwater contamination and escalate GHG emissions (IPCC, 2014). Given these significant environmental impacts, there is an urgent need to explore sustainable alternatives that can reduce the reliance on synthetic nitrogen fertilizers. One such promising alternative is asymbiotic nitrogen fixation (ANF), which involves diazotrophic bacteria forming associations with non-legume crops like maize.

Asymbiotic Nitrogen Fixation

Asymbiotic nitrogen fixation (ANF) is especially noteworthy, where non-legume crops form associations with diazotrophic bacteria in the rhizosphere, either on the root surface or within the roots (de Bruijn, 2015). These root-associated, nitrogen-fixing bacteria can potentially supply significant amounts of nitrogen to cereal crops, thereby decreasing reliance on synthetic nitrogen fertilizers.

Microbial cells in ANF demonstrate remarkable adaptability, thriving independently in diverse environments including the soil, terrestrial, and aquatic ecosystems. Recent studies suggest that ancient lines of cereals might have benefitted from nitrogen-fixing mutualistic bacteria, as seen in landrace maize varieties by Van Deynze et al. (2018). This adaptability makes ANF a viable option for improving nitrogen utilization efficiency in various agricultural settings.

Asymbiotic nitrogen fixation offers direct and sustainable nitrogen sources for cereal crops, promising improvements in nitrogen utilization efficiency, reduced environmental impacts, and long-term sustainability in cereal crop production (Kennedy & Islam, 2001). Microbial nitrogen fixation plays a critical role in enriching soil fertility and could minimize the chemical N fertilizers inputs in agriculture systems (Stagnari et al., 2017; Kebede, 2021). However, the effectiveness of root-associated diazotrophs depends on their nitrogenase activity, which is energetically costly, requiring 16 ATP per reduced N_2 molecule (Curatti & Rubio, 2014).

Moreover, the activity of these diazotrophs is limited in high nitrogen conditions, which can inhibit their nitrogen-fixing capabilities. This limitation underscores the importance of optimizing conditions for ANF to maximize its benefits (Smercina et al. 2019). Despite these challenges, ANF represents a promising avenue for sustainable agriculture, offering a potential

solution to the environmental and economic drawbacks associated with synthetic nitrogen fertilizers.

Genetic Modifications for Enhanced BNF

In addressing the limitation of biological nitrogen fixation, it becomes essential to consider the genetic modifications of microbes (Dixon & Kahn, 2004). Gene-editing techniques have opened new possibilities for enhancing the relationship between diazotrophs and plant roots, particularly in nitrogen-rich soil conditions that would normally inhibit nitrogen fixation (Bloch, Ryu, et al., 2020). These advancements in microbial engineering aim to improve the nitrogen-fixing capabilities of diazotrophic bacteria, thereby providing a more sustainable alternative to synthetic fertilizers.

Recent developments in microbial engineering have led to the creation of genetically modified strains designed to enhance nitrogen fixation. One notable commercial example is Proven®, a bioinoculant that has shown promise in enhancing nitrogen-fixing capabilities. Proven® contains genetically modified strains of diazotrophic bacteria, such as *Klebsiella variicola* and *Kosakonia sacchari*, which have been optimized to improve their interaction with plant roots and their nitrogen-fixing efficiency (Wen et al., 2021). These modifications aim to overcome the natural suppression mechanisms triggered by high nitrogen levels soils, allowing the bacteria to continue fixing nitrogen even in nitrogen-rich conditions (Bloch, Clark, et al., 2020).

Strategies to Enhance Nitrogen Use Efficiency in Cereal Crop Production

The shift of the agriculture sector towards sustainability emphasizes the significance of Nitrogen Use Efficiency (NUE). Defined as a metric for evaluating and comparing nitrogen management practices, NUE is a crucial tool for environmental and economic goals (Novoa &

Loomis, 1981; Congreves et al., 2021). Enhancing NUE aims to reduce nitrogen losses, optimize environmental benefits, and foster sustainable agriculture practices (Fixen & West, 2002).

Understanding NUE is deeply intertwined with effective fertilizer management and the interaction between soil, water, and plant systems, which are key to sustainable agricultural intensification (Fixen & West, 2002). Several methodologies exist for calculating NUE, each with specific advantages and inherent limitations. Dobermann (2007) notes that these methods range from field-based evaluations to more complex computational models. While each approach offers valuable insights, it is recognized that relying on a single method may not fully capture the intricate dynamics of nitrogen use. Therefore, a comprehensive assessment requires multiple perspectives.

However, achieving high NUE is challenged by various factors, including leaching, runoff, and denitrification, which contribute to the loss of reactive nitrogen and exacerbate the nitrogen load in ecosystems (Swaney et al., 2012). In response to these challenges, innovative solutions like bioinoculants have gained attention for their potential to boost plant nitrogen nutrition and growth, thereby enhancing NUE. Bioinoculants are a diverse group of beneficial microorganisms, including bacteria, fungi, and other soil microbes, which serve as an alternative to synthetic nitrogen fertilizers (Dobbelaere et al., 2001; Vessey, 2003). They significantly enhance nitrogen use efficiency by improving plant nitrogen uptake and utilization, leading to reduced nitrogen losses and lower environmental impacts (Adesemoye & Kloepper, 2009).

For instance, the inoculation of diazotrophs such as *P. cypripedii* and *K. arachidis* has been shown to increase plant nitrogen content significantly, as corroborated by ^{15}N isotope dilution analysis (Singh et al., 2021). These findings suggest that *P. cypripedii* and *K. arachidis* are beneficial endophytes that could be used as biofertilizers to improve plant nitrogen nutrition and growth, particularly in crops like sugarcane. By enhancing the nitrogen-fixing capabilities of root-

associated diazotrophic bacteria, bioinoculants like Proven® might reduce the dependency on synthetic nitrogen fertilizers, thereby mitigating their environmental impacts and contributing to greater NUE (Davis & Rice, 2021; Woodward, 2023).

There is significant potential to use nitrogen-fixing microbial inoculants to enhance the levels of biologically-fixed nitrogen in agricultural systems (Bottomley & Myrold, 2007). These biological agents promote plant growth, improve soil structure, and increase crop resilience against biotic and abiotic stresses (Yang et al., 2009; Bashan et al., 2014). Consequently, bioinoculants represent a promising strategy for achieving sustainable agriculture by improving NUE and reducing the reliance on synthetic fertilizers (Kennedy, 2004; Adesemoye et al., 2009).

This literature review underscores the promise of associative nitrogen fixation in maize cultivation, highlighting the need for further research to optimize these biological processes for improved crop yields and sustainable agriculture. The studies mentioned provide a foundation for exploring the potential of diazotrophs in maize and other cereal crops, particularly focusing on the conditions that maximize their nitrogen-fixing capabilities. Future research in this area could lead to more sustainable agricultural practices, reducing reliance on synthetic fertilizers and it's the environmental impacts.

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Chapter 2 - Investigating the Role of Rhizospheric Bacteria Diazotrophs in Enhancing Corn Nitrogen Use Efficiency

Introduction

Nitrogen (N) is a critical macronutrient for maize (*Zea mays* L.), driving essential physiological processes such as photosynthesis and protein synthesis (Hirel et al., 2005). Annually, U.S. farmers apply approximately 12.3 billion pounds of nitrogen to maize fields (USDA, 2021), underscoring nitrogen's importance in achieving high yields. However, nitrogen use in agriculture is often inefficient, with only 40-50% of the total organic and inorganic N applied each crop season being absorbed by crops (Mosier et al., 2002). This inefficiency leads to

substantial environmental impacts, including groundwater contamination and increased greenhouse gas emissions (Bundy & Andraski, 2005; IPCC, 2014).

The loss of synthetic nitrogen fertilizers arises from multiple factors, including volatilization, leaching, and denitrification. Ammonium (NH_4^+), a common form of synthetic nitrogen, is prone to volatilization into ammonia gas (NH_3), particularly when not adequately incorporated into the soil (Rochette et al., 2013). Nitrate (NO_3^-) is susceptible to leaching and can undergo microbial conversion into gas forms like nitrous oxide (N_2O) and dinitrogen (N_2) under anaerobic conditions, further contributing to nitrogen loss (Suri et al., 2021). These losses not only represent significant economic costs for farmers but also pose serious environmental risks, exacerbating issues such as eutrophication and climate change.

Given these challenges, improving nitrogen use efficiency (NUE) has become a critical focus in agriculture (Galloway et al., 2008; Snyder et al., 2014). The NUE measures how effectively plants utilize available nitrogen and is a key indicator of sustainable agricultural practices (Cassman, 1999). Traditional approaches to enhancing NUE have centered on optimizing fertilizer application rates and timing. However, recent advancements have introduced biological solutions, such as bioinoculants, which offer new avenues for sustainable nitrogen management.

Bioinoculants like Proven® leverage the nitrogen-fixing capabilities of diazotrophic bacteria to reduce reliance on synthetic fertilizers. Advances in DNA sequencing and gene-editing technologies have significantly enhanced our understanding of these bacteria, revealing their potential to improve nitrogen uptake in maize (Barrangou & Doudna, 2016; Wen et al., 2021). For example, the identification of specific diazotroph strains, such as *Klebsiella variicola* strain 137-1036, marks a significant breakthrough in harnessing bacterial nitrogen fixation for agricultural benefit (Wen et al., 2021). These strains, including genetically engineered microbes like Proven®,

have been modified to continuously fix nitrogen, offering a sustainable alternative to synthetic fertilizers (Bloch, Ryu, et al., 2020; Bloch, Clark, et al., 2020).

Despite the promise of bioinoculants, challenges remain in integrating them into existing agricultural practices, particularly in regions with varying environmental conditions or crop types. This study focuses on evaluating the impact of Proven® on nitrogen uptake, dry matter accumulation, and grain yield in maize under field conditions. By assessing these parameters at critical developmental stages, this research aims to provide a comprehensive understanding of how bioinoculants can enhance nitrogen dynamics throughout the growing season. Additionally, this study explores the extent to which Proven® influences nitrogen remobilization, offering insights into its potential to reduce reliance on synthetic fertilizers.

We hypothesized that inoculation with diazotrophic bacteria will enhance nitrogen uptake and efficiency, allowing further reduction in synthetic nitrogen inputs without compromising crop productivity. To test this hypothesis, the study will address two primary objectives: (i) to assess the effect of Proven® on nitrogen use efficiency, and (ii) to evaluate its effects on grain yield, dry matter production, and nitrogen uptake in maize. The findings from this research will contribute to the development of more sustainable nitrogen management strategies, potentially offering both economic and environmental benefits.

Materials and Methods

Experimental site description and treatments

The study was conducted in a rainfed area, continuous corn, and no-till system at the Kansas State University Agronomy North Farm (39°12'19.1"N, 96°35'43.9"W) over three growing seasons in 2021 (2 June to 17 September), 2022 (16 June to 26 October), and 2023 (25 April to

September 8). The planting density was consistent across the three years (66,900 seeds ha^{-1}), with different hybrid types used each year: 1089AM in 2021, DKC 64-65RIB (VT2PRIB) in 2022, and DKC 64-65 (VT2P) in 2023. The soil is characterized as well-drained Kennebec silt loam (fine-silty, mixed, superactive, mesic Cumulic Hapludolls) (Survey Staff, 2022). The region has a humid continental climate (Cfa); with an average annual rainfall of 800 mm and an average temperature of 11.4 °C. Fig.1.1 shows the accumulated monthly rainfall and mean monthly temperature throughout the crop season.

The experiment followed a split-plot randomized complete block design (RCBD) with four nitrogen (N) rates (0, 56, 112, and 168 kg N ha^{-1}) as the main plot treatments, applied as urea. The bacterial inoculant used in this research was Proven® [*Kosakonia sacchari* and *Klebsiella variicola*], characterized as the subplot treatment, which was applied in-furrow at planting. The experiment consisted of six replications. Herbicide was used for weed control as needed.

Collection and analysis

Soil samples were collected before the corn growing season and throughout its duration, while biomass samples were collected at eight-leaf (V8), tasseling (VT), and harvest (R6) corn stages. Whole shoot samples were taken randomly from each plot by cutting five plants at each stage. These samples were then dried in a forced air circulation oven at 65°C, ground in a Willey mill, and sieved to pass through a 2mm mesh. The samples were analyzed by dry combustion using a C-N elemental analyzer (Flash EA 1112 Series Thermo Finnigan Italia S.p.A., MI, Italy) (Mikha & Rice, 2004; Rice et al., 2021; Lin et al., 2023). The initial results were generated as percentages, which were subsequently converted to kg ha^{-1} for further calculations.

Grain and stover yield were estimated by harvesting two rows (3 m each) from the right of each planting plot. Ears were weighed, shelled, and grain weighed for yield. Grain yield was expressed on a 15.5% moisture basis.

Soil samples were collected from three layers (0-5, 5-15, and 15-30 cm) at the same stages as biomass samples. The chemical characteristics of the soil (pre-plant) including pH, phosphorus (P), and potassium (K), were analyzed by the Soil Testing Lab at Kansas State University and are presented in Table 1.

Soil samples collected throughout the growing season were used to determine NO_3^- and NH_4^- concentrations. They were determined by KCl extraction: 25 g of moist soil was placed in an Erlenmeyer flask with 100mL 1 M KCl, and shaken on an orbital shaker at 300 rpm for 1 hour (Keeney & Nelson, 1982). The solution was then filtered through Whatman #42 filter paper.

Efficiency Calculations

The N use efficiency indices in this study included were, categorized into groups based on fertilizer and plant tissue (Congreves et al., 2021). The calculations were based on the following equations:

(1) $$\text{NAE (kg grain kg N}^{-1}) = \frac{Yieldf - Yield0}{Fertilizer\ N}$$

Where NAE is the nitrogen agronomic efficiency; yieldf is the grain yield (kg ha^{-1}) of the fertilized plots, yield0 (kg ha^{-1}) is the grain yield of unfertilized plots, and fertilizer N (kg ha^{-1}) is the amount of N applied (kg ha^{-1}).

(2) $$\text{NRE (kg N kg N}^{-1}) = \frac{\Delta\ N\ Uptake}{Fertilizer\ N}$$

Where NRE is the nitrogen recovery efficiency; Δ N uptake is the difference between the total content N rate (kg N ha^{-1}) of the fertilized plots and the unfertilized plots, and fertilizer N (kg N ha^{-1}) is the amount of N applied (kg N ha^{-1}).

(3) $$\text{IE (kg grain kg N uptake}^{-1}) = \frac{Yield}{N\ uptake}$$

Where IE is the internal utilization efficiency of a nutrient; yield is the grain yield (kg ha^{-1}) of the fertilized plots and unfertilized plots, and N uptake is the total N content from the fertilized and unfertilized plots (kg N ha^{-1}).

(4) $$\text{PFP (kg grain kg N}^{-1}) = \frac{Yield}{Fertilizer\ N}$$

Where PFP is the partial-factor productivity; yield is the grain yield (kg ha^{-1}) of the fertilized plots, and fertilizer N is the amount of N applied (kg N ha^{-1}).

Nitrogen uptake and Remobilized N

Nitrogen uptake was determined by quantifying the amount of nitrogen present in the plant. The N uptake was calculated using the following equation:

(5) $$\text{N uptake (kg N ha}^{-1}) = Biomass \times N\ concentration$$

Where *Biomass* represents the dry weight of the plant material collected, and the *N concentration* is the percentage of nitrogen determined from leaf tissue analysis.

Remobilized N refers to nitrogen that is translocated from vegetative plant parts to the grain after flowering. To calculate the N remobilization contribution to grain N content, we first determined the post-flowering N uptake:

(6) $$\text{Post-flowering N uptake (kg N ha}^{-1}) = Total\ N\ uptake\ at\ R6 - N\ uptake\ in\ plant\ at\ R1$$

Here, the *total N uptake at R6* represents the total plant and grain N at physiological maturity, while the *N uptake in the plant at R1* denotes the total plant nitrogen at the beginning of the reproductive stage (silking). The N contribution to the grain from remobilization was then determined:

(7) N contribution from remobilization (kg N ha^{-1}) $= N\ uptake\ in\ grain\ at\ R6 - Post\ flowering\ N\ uptake$

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using type III sums of squares to assess the significance of the fixed effects (R Core Team, 2019). A linear mixed-effects model (lmer) was applied to investigate the relationship between treatments, incorporating random effects for block and treatment interactions to account for within-block variability.

Post-hoc comparisons were performed using estimated marginal means (emmeans) to further explore significant effects identified by the ANOVA. Pairwise comparisons were obtained using the compact letter display (cld) function to identify differences between treatment combinations of nitrogen rate and the "proven" variable.

Additionally, a generalized linear model (GLM) with a Gamma error distribution was employed to analyze yield data, including fixed effects for block, nitrogen rate, treatment, and year, with Type III sums of squares used to assess the significance of these factors.

Results

Whole plant biomass

Throughout the 2021 season, the interaction between the N rate and inoculant affected plant dry matter at the V8 growth stage of corn (Table 2). Specifically, dry matter was higher with Proven® than without, with an increase of 334 kg ha^{-1} at 56 kg N ha^{-1} and 61 kg ha^{-1} at 112 kg N ha^{-1} (Figure 2). Additionally, N rate significantly affected the V8, VT, and R6 growth stages in 2021 and 2022.

In 2022, notably, at the VT growth stage, both the N rate and Proven®, along with their interaction, significantly affected dry matter (Figure 3, Table 2). Plants treated with Proven® had greater dry matter (±1516 kg ha^{-1}) in unfertilized plots (Table 2). At the R6 growth stage, Proven® also outperformed at 0 kg N ha^{-1} and 112 kg N ha^{-1}, with increases of 431 kg ha^{-1} and 202 kg ha^{-1}, respectively (Table 2), however, it was not statistically significant. Lastly, N rate significantly affected plant dry matter at all evaluated growth stages (Table 2).

In 2023, during the VT growth stage, the application of Proven® increased dry matter production compared to the untreated (non-Proven®) plots. As illustrated in Figure 4, the average dry matter for plots treated with Proven® was 8106 kg ha^{-1}, while the untreated plots averaged 7539 kg ha^{-1}.

N uptake

Across the 2021, 2022, and 2023 growing seasons, N uptake in corn was critically analyzed at key developmental stages. These observations are graphically detailed in Figure 5 and statistically summarized in Table 3.

In 2021, N rate significantly affected N uptake at the V8 stage, with an interaction between Proven® and N rate. Proven® augmented N uptake by 6.3 kg N ha^{-1} at 56 kg N ha^{-1} and by 4.1 kg N ha^{-1} at 112 kg N ha^{-1} compared to untreated plots (Table 3, Figure 6). The VT stage continued this trend, indicating a significant effect from both the N rate and the interaction with Proven® (Figure 7). At the R6 stage, even though Proven® had a better performance at 0, 56, and 168 kg N ha^{-1}, this was not significant, and the effect was solely governed by the N rate (Table 3).

In 2022, at the VT stage, the effect of Proven® was statistically significant, along with its interaction with the N rate (Table 3). Nevertheless, treated plots only outperformed the untreated

at 0 kg N ha^{-1}, with an N uptake increase of 43 kg N ha^{-1}. Additionally, N uptake across all stages was affected by N rate (Table 3).

In 2023, Proven®-treated corn had increased N uptake at the V8 growth stage compared to untreated plots, with gains between 2.9 to 10.7 kg N ha^{-1} across the 4 N rates (Table 3). At the VT stage, the benefits of Proven® treatment were more pronounced, with marked increases in N uptake of 20.6 to 81.1 kg N ha^{-1} across the N rates over the untreated plots (Table 3). At the R6 stage, treated plots had a moderate increase in N uptake of 7 kg N ha^{-1} only at 112 kg N ha^{-1} compared to untreated plots. Although these increases were not statistically significant (Table 3), they suggest a consistent advantage in nutrient acquisition with Proven®.

Remobilized N

Our analysis revealed that in Proven®-treated corn in 2023, the grain protein content averaged 18.4% at the highest N rate, compared to 11.7% without Proven® (supplementary material). Specifically, at 168 kg N ha^{-1}, Proven® treated corn had 84 kg N ha^{-1} from remobilized sources and 18 kg N ha^{-1} from post-flowering absorption, totaling 102 kg N ha^{-1} in the grain (Figure 8). Conversely, without Proven®, 35 kg N ha^{-1} was from remobilization and 76 kg N ha^{-1} was from post-flowering absorption out of 111 kg N ha^{-1} in the grain (Figure 9). This indicates that 82% of the grain protein content with Proven® was derived from remobilized nitrogen, while 18% came from post-flowering uptake (Figure 10). In contrast, for corn without Proven®, 32% of the grain protein content was from remobilized nitrogen and 68% from post-flowering uptake (Figure 11).

Yield and its components

Over the three-year study, the effects of nitrogen application rates and Proven® treatment on corn grain yield were assessed (Figure 12), revealing nuanced effects across growing seasons

and treatments. The shift from 2021 to 2022 had a substantial increase in yield ($P < 0.0001$). Conversely, the comparison between 2021 and 2023 yielded no statistically significant difference in yield ($P = 0.989$). Additionally, the Proven® treatment did not have a statistically significant effect on grain yield when comparing treated versus untreated groups.

Nitrogen Agronomic Efficiency (NAE)

In 2021, nitrogen agronomic efficiency (NAE) was not significantly affect by nitrogen (N) rate, Proven®, or their interaction (Table 4, Figure 13). In 2022, a notable effect of N rate on NAE was observed, while Proven® and its interaction with N rate were not significant (Table 4, Figure 14). In 2023, similar to 2021, NAE was not significantly affected by the N rate, Proven® treatment, or their interaction (Table 4, Figure 15).

Nitrogen Recovery Efficiency (NRE)

In 2021, the nitrogen recovery efficiency (NRE) had no discernible effect from the N rate, Proven®, or the interaction between Proven® and N rate (Table 4, Figure 16). In 2022, NRE was sensitive to the N rate but was unaffected by Proven® and its interaction with N rate (Table 4, Figure 17). In 2023, NRE was significantly affected by N rate, with no significant effects from Proven® or the interaction between Proven® and the N rate (Table 4, Figure 18).

Internal Efficiency (IE)

In 2021, internal efficiency (IE) was not significantly affected by the N rate, Proven® treatment, or their interaction (Table 4, Figure 19). In 2022, IE showed no statistically significant impact from the N rate, Proven® treatment, or their interaction (Table 4, Figure 20). In 2023, IE was significantly influenced by both the N rate and Proven®, with a significant interaction between the N rate and Proven® (Table 4, Figure 21).

Partial Factor Productivity (PFP)

In 2021, partial factor productivity (PFP) had a significant response to the N rate, while Proven® had no significant effect, and the interaction between Proven® and N rate was also not significant (Figure 22). In 2022, PFP was significant to N rate, with a marginal effect from Proven®, and no significant interaction between Proven® and the N rate (Figure 23). Continuing this pattern in 2023, PFP significantly responded to the N rate, with Proven® having no significant effect and no significant interaction between Proven® and the N rate (Figure 24).

Discussion

Dry Matter

Our findings suggest a stage-specific response to Proven®, reflecting the dynamic nature of plant-microbial interactions across different phases of plant development (Edwards et al., 2018). For instance, from the three stages measured, the effect of Proven® or its interaction with N rate was more pronounced in the vegetative stages (V8 and VT). This was particularly evident in 2023 at the V8 stage, where Proven® led to greater dry matter across all N rates (Table 2).

At the VT stage, the benefits of the bioinoculant became more apparent, as highlighted in Figure 4. While no significant interaction between Proven® and N rate was observed, Proven®-treated plants tended toward increased dry matter ($p = 0.062$) compared to untreated plants when averaged across all nitrogen rates. This aligns with periods of rapid growth, including shoot elongation, increased plant height, stalk diameter, and leaf number, which are often enhanced by inoculation (Baral & Adhikari, 2014; Ferrarezi et al., 2022; Carvalho et al., 2023).

The visual evidence from the supplemental material reinforces that the primary benefits of Proven® manifested earlier in the plant's lifecycle, particularly at critical growth stages such as VT. As research has shown, plant developmental stages significantly impact the abundance of

beneficial microbes, such as Rhizobium, which peaks at the tasseling stage in maize (Xiong et al., 2024).

N Uptake

Similar to dry matter, nitrogen uptake with Proven® across all three seasons had significant interactions with the nitrogen (N) rates, particularly by the VT stage (Table 3). Notably, in 2023, there was a consistent increase in nitrogen uptake at the VT stage across all N rates with Proven®, except in the absence of added nitrogen (0 kg N ha^{-1}). This increase is likely due to the enhanced nitrogen fixation capabilities of diazotrophic bacteria in the inoculant, suggesting that more than 50 kg ha^{-1} of nitrogen was fixed at the VT stage at the highest N rate. Conversely, the ineffectiveness of Proven® in the 0 kg N ha^{-1} plots may be due to N-deficient plants' inability to produce sufficient root exudates to support effective root colonization by the bacteria (Carvalhais et al., 2011).

The release of root exudates, crucial for nutrient uptake and fostering beneficial soil microbial communities, is influenced by soil nutrient availability. High nutrient availability promotes the secretion of root exudates, enhancing these interactions. Conversely, low nutrient availability limits the plant resources for root exudates, potentially compromising nutrient acquisition and plant-microbe relationships (Y. Liu et al., 2022; Y. Li et al., 2023). In arid and nutrient-poor ecosystems, microbial competition for nitrogen becomes intense, affecting plant nutrient uptake (Cui et al., 2020). Additionally, microorganisms can compete with plant roots for nutrients under nutrient-deficient conditions (Jing et al., 2020).

In 2023, early planting likely advanced the vegetative and reproductive growth stages of corn, aligning key growth stages with periods of better rainfall distribution, particularly during the VT stage (Figure 1). These favorable conditions likely enhanced the efficacy of Proven, as

suggested by the significantly improved nitrogen uptake in 2023 with treated plots compared to untreated ones (Table 2). Environmental factors such as climate, temperature, and precipitation patterns significantly influence crop-associated diazotrophic communities and their interactions with plants (Xiong et al., 2024).

Furthermore, the variation in temperature and rainfall can change the behavior and relationships of certain bacteria, which may explain why Proven® performed better in 2023. Low soil moisture and limited carbon availability may cause strong microbial carbon limitation during early succession (Cui et al., 2020). Changes in soil characteristics result in alterations to root exudates and subsequently the rhizobiome composition (Vives-Peris et al., 2018). Additionally, certain bacteria have been shown to enhance plant tolerance to biotic stresses (Santhanam et al., 2015) and abiotic stresses (Rolli et al., 2015). For instance, increased temperatures alter root exudates to favor beneficial bacteria that enhance crop survival (Ali et al., 2011). Leveraging these beneficial interactions between the root and rhizosphere can enhance crop resilience to various stresses (Ali et al., 2011).

Yield

Overall, the lack of significant effect of Proven® treatment on yield underscores its minimal impact on enhancing yield across the study period, in contrast to the marked influence of yearly environmental conditions and nitrogen rates. These findings align with those reported by Davis & Rice (2021) at the same site in Kansas, where Proven® revealed no effect on yield over three years. This highlights the complex interplay between nitrogen application, treatment interventions, and temporal factors in determining corn grain yield.

However, some studies have demonstrated the potential for yield increases with the use of Proven®. Woodward (2023) observed that increases in plant N accumulation driven by Proven®

were correlated with higher grain yields, particularly at intermediate nitrogen fertilizer rates when averaged across all N rates. Similarly, Wel et al. (2001) found that Proven® consistently provided a yield advantage and reduced yield variance across years. This was attributed to the stable performance of free-living diazotrophs in corn roots across different geographies, likely due to the consistent microclimate in the rhizosphere.

Nitrogen use efficiencies

Nitrogen use efficiency (NUE) is a critical metric for optimizing nutrient management in agriculture and is frequently used as a benchmark in studies evaluating sustainable farming practices. However, several limitations in NUE calculations may have influenced the evaluation of Proven® in this study. For instance, standard NUE metrics typically omit nitrogen inputs from non-fertilizer sources like atmospheric deposition, nitrogen fixation, and the mineralization of (SOM) (Godinot et al., 2014). Moreover, NUE assumes that soil organic matter (SOM) remains constant from year to year (Schröder et al., 2003), which may lead to the misinterpretation of nitrogen losses if crops increasingly rely on mineralized SOM over time. This underscores the potential for skewed NUE results in systems where SOM is dynamic, potentially limiting the efficacy of bioinoculants like Proven®.

In this study, nitrogen uptake and yield were measured at multiple stages of crop development, though the NUE calculations relied solely on data from the R6 (mature) stage. While this approach aligns with conventional methods, it may obscure Proven's early-stage efficacy. In fact, nitrogen uptake with Proven® showed greater efficiency during earlier growth stages, such as the VT stage in 2023 (Supplemental material, Figure 2). However, because NUE metrics focused on later stages, this early-stage advantage may not have been fully captured, contributing to the lack of significant overall NUE improvement. Such discrepancies have been noted in other studies,

such as Zeffa et al. (2019), who reported similar challenges in capturing the full impact of bioinoculants on NUE when measurements are restricted to later growth stages. To account for these dynamics, future studies should include NUE assessments across various growth stages to provide a more comprehensive understanding of bioinoculant efficacy.

Additionally, the relationship between nitrogen uptake and yield offers further insight into the performance of Proven®. For example, in 2022, higher nitrogen uptake and yields were observed compared to 2023, particularly at the 112 kg N ha^{-1} rate with Proven®. Despite these pronounced values, the results were not statistically significant. This could be attributed to several factors, including variability in environmental conditions and the inherent complexities of NUE calculations (Dobermann et al., 2007). For instance, in 2023, nitrogen recovery efficiency (NRE) was higher than in 2022, even though nitrogen uptake was lower. This paradox can be explained by the low nitrogen uptake observed at the 0 N rate in 2023, which magnified the efficiency values when calculated as the difference between control and nitrogen-treated plots. Conversely, in 2022, the relatively higher nitrogen uptake at the 0 N rate contributed to lower NRE values at the 168 N rate, despite higher overall uptake.

Despite these challenges, numerous studies have demonstrated the potential benefits of microbial inoculants in enhancing nutrient efficiency and crop productivity. For instance, J. Li et al. (2022) found that microbial inoculants improved crop yields by an average of 22.25%, primarily by enhancing nutrient uptake and alleviating environmental stressors. While Proven® did not significantly impact overall NUE in this study, its positive impact on nitrogen uptake and early-stage efficiency suggests potential within an integrated nutrient management strategy. Further research, incorporating comprehensive growth-stage measurements and varying environmental

conditions, will be crucial for fully understanding the role of bioinoculants like Proven® in modern agriculture systems.

Remobilized N

In 2023, a detailed assessment of the nitrogen content in grain focused on distinguishing remobilized nitrogen from vegetative tissues and the uptake of nitrogen post-flowering. Proven®-treated plants exhibited higher N uptake at certain growth stages, though not statistically different. The N uptake post-flowering was calculated as the difference between total N uptake at the R6 stage and the N uptake in the plant at the R1 stage. The N contribution to the grain from remobilization was determined by subtracting this post-flowering N uptake from the total grain N uptake (Debruin & Butzen, 2014; Chen et al., 2015). The nitrogen immobilization data illustrates that most of the nitrogen content in the grain with Proven® came from the tissues. This indicates that the plants relied heavily on the nitrogen taken up by the VT stage but were unable to maintain this uptake trend until the end of the season. This could have affected the yield and, consequently, the NUE, potentially explaining the lack of gains with Proven® in terms of NUE metrics. Studies by Martin et al. (2005) and Ciampitti & Vyn (2012) have similarly highlighted the importance of sustained nitrogen uptake throughout the growing season for optimal NUE and yield outcomes.

The non-significant effect of Proven® on yield and NUE indices aligns with the notion that these aspects are multifactorial and not solely dependent on microbial inoculation. This suggests a potential mechanism by which Proven® might improve nitrogen use efficiency under specific conditions, highlighting the need for further research to explore these dynamics in different crop species and stages of growth. This observation is supported by studies from Chaudhary & Shukla. (2018), Lee Díaz et al. (2021) and Orozco-Mosqueda et al. (2021), which found that bioinoculant

performance is highly context-dependent, varying with environmental conditions like pH, humidity, available nutrients, as well as crop types and management practices.

Conclusions

The findings revealed that while Proven® had a stage-specific influence on dry matter accumulation and nitrogen uptake, particularly at the vegetative (V8) and tasseling (VT) stages, its overall impact on grain yield and NUE indices was limited. However, the environmental benefits associated with the use of Proven® highlight its potential as a sustainable agricultural practice.

Key Findings:

Dry Matter Accumulation:

Proven® demonstrated a significant increase in dry matter at various nitrogen (N) rates, especially at the V8 and VT stages, indicating potential benefits during the early and mid-growth stages of corn.

Nitrogen Uptake:

Enhanced nitrogen uptake was observed with Proven® treatment, particularly at the VT stage in 2023. However, the absence of added nitrogen in some plots limited the effectiveness of Proven®, likely due to insufficient root exudates for effective microbial colonization.

Nitrogen Use Efficiency (NUE):

The study highlighted the critical role of nitrogen rate in determining NUE, with significant interactions observed for internal efficiency (IE) in 2023. However, Proven® did not consistently enhance NUE metrics, possibly due to methodological limitations in measuring NUE only at the R6 stage and not considering earlier growth stages where Proven® showed potential benefits.

Grain Yield:

The impact of Proven® on grain yield was not significant across the three years, aligning with findings from previous studies. This underscores the multifactorial nature of yield outcomes, which are influenced by a combination of environmental conditions, management practices, and microbial inoculation.

Remobilized Nitrogen:

The analysis of nitrogen immobilization data in 2023 indicated that most of the nitrogen content in the grain with Proven® came from remobilized tissue nitrogen. This suggests that the plants relied heavily on the nitrogen taken up by the VT stage but could not maintain this uptake trend until the end of the season. This reliance on early uptake potentially affected the yield and NUE, highlighting the need for strategies to sustain nitrogen uptake throughout the growing season.

While Proven® demonstrated promise in enhancing dry matter and nitrogen uptake at specific growth stages, its overall impact on NUE and grain yield was limited in this study. However, the environmental benefits of using Proven® highlight its potential as a sustainable agricultural practice. The interconnectedness of climate, soil conditions, and agricultural biotechnology underscores the need for integrated approaches to improve nutrient use efficiency and crop productivity.

Future research should focus on understanding the complex interactions between bio-inoculants, environmental factors, and crop physiology to develop effective and sustainable agricultural practices. We can move towards more efficient and environmentally friendly farming systems that meet the growing global food demand by addressing these challenges.

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Table 1. The main features of the soil in the pre-plant stage.

Soil characteristics	2021	2022	2023
	0-10 (cm)	0-10 (cm)	0-10 (cm)
Available P (Extracted by Mehlich-1) (mg kg^{-1})	28.0	24.2	33.2
Exchangeable K (Extracted by Mehlich-1) (mg kg^{-1})	258	262	247
pH in water	7.2	7.2	7.18

Table 2. Dry matter (kg ha^{-1}) in corn at V8, VT, and R6 growth stages for the years 2021, 2022, and 2023.

	Dry matter - V8		**Dry matter - VT**		**Dry matter - R6**	
	Proven®	No Proven®	Proven®	No Proven®	Proven®	No Proven®
N rates			**2021**			
0	1575	1578	9491	10133	4691	4914
56	2033	1699	10160	10252	5802	5540
112	1933	1872	10166	11851	6174	6341
168	1859	2291	11524	14621	6793	6626
			P- values			
Proven®	0.983		0.585		0.517	
Rates	<0.001***		<0.001***		<0.001***	
P*R	0.01*		0.285		0.689	
			2022			
0	2719	2455	8146	6630	6003	5572
56	2929	3648	8103	8910	7334	7531
112	3035	3134	8378	8485	7564	7362
168	2740	2891	7266	8635	8216	8464
			P- values			
Proven®	0.369		0.03*		0.93	
Rates	<0.001***		0.002**		<0.001***	
P*R	0.125		0.01*		0.94	
			2023			
0	729	517	8225	6714	3470	3398
56	1416	1233	8468	7880	6046	6554
112	1504	1178	8139	7224	6359	5993
168	1438	1065	7591	8335	7089	7891
			P- values			
Proven®	0.2		0.06.		0.93	
Rates	<0.001***		0.19		<0.001***	

P*R	0.83	0.23	0.72

Table 3. N uptake (kg ha^{-1}) in corn at V8, VT, and R6 growth stages for the years 2021, 2022, and 2023.

	N uptake - V8		N uptake - VT		N uptake - R6	
	Proven®	No Proven®	Proven®	No Proven®	Proven®	No Proven®
N rates			**2021**			
0	30	30.6	89	87.1	49.6	48.9
56	45.6	39.3	108.3	97.7	82.2	79.6
112	50.8	46.7	93.4	155	97.4	101
168	47.1	64.8	140	210	104	98.1
			P- values			
Proven®		0.92		0.68		0.94
Rates		<0.001***		<0.001***		<0.001***
P*R		0.02*		0.02*		0.9
			2022			
0	50.6	49.6	115	72.0	78.5	79.5
56	66.2	84.1	111	139	134	134
112	74.4	77.0	144	168	161	158
168	69.1	73.9	181	193	180	187
			P- values			
Proven®		0.89		0.009**		0.93
Rates		<0.001***		<0.001***		<0.001***
P*R		0.31		0.003**		0.94
			2023			
0	15.4	10.9	45.1	47.2	39.5	47.4
56	36.2	33.3	92.3	71.7	71.7	72.8
112	47.5	37.5	121	96.0	98.3	91.3
168	47.3	36.6	150	98.7	167	175
			P- values			
Proven®		0.4		0.9		0.4
Rates		<0.001***		0.004**		<0.001***
P*R		0.62		0.14		0.60

Table 4. ANOVA results for nitrogen use efficiency (NUE) variables across different years.

NUE	N rate	Proven	N rate: Proven
		2021	
IE	0.292	0.537	0.964
NAE	0.134	0.425	0.797

NRE	0.300	0.766	0.881
PFP	< 0.001***	0.429	0.659
	2022		
IE	0.708	0.962	0.708
NAE	< 0.0001***	0.125	0.418
NRE	0.006**	0.957	0.900
PFP	< 0.0001***	0.09.	0.307
	2023		
IE	< 0.0001***	< 0.001**	0.02*
NAE	0.362	0.463	0.577
NRE	< 0.0001***	0.796	0.554
PFP	< 0.001***	0.405	0.493

The table shows the p-values for the effects of Nitrogen rate, Proven, and their interaction (N rate: Proven) on four Nitrogen Use Efficiency (NUE) metrics: Nitrogen Agronomic Efficiency (NAE), Nitrogen Recovery Efficiency (NRE), Internal Efficiency (IE), and Partial Factor Productivity (PFP). Significant p-values are marked with asterisks: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

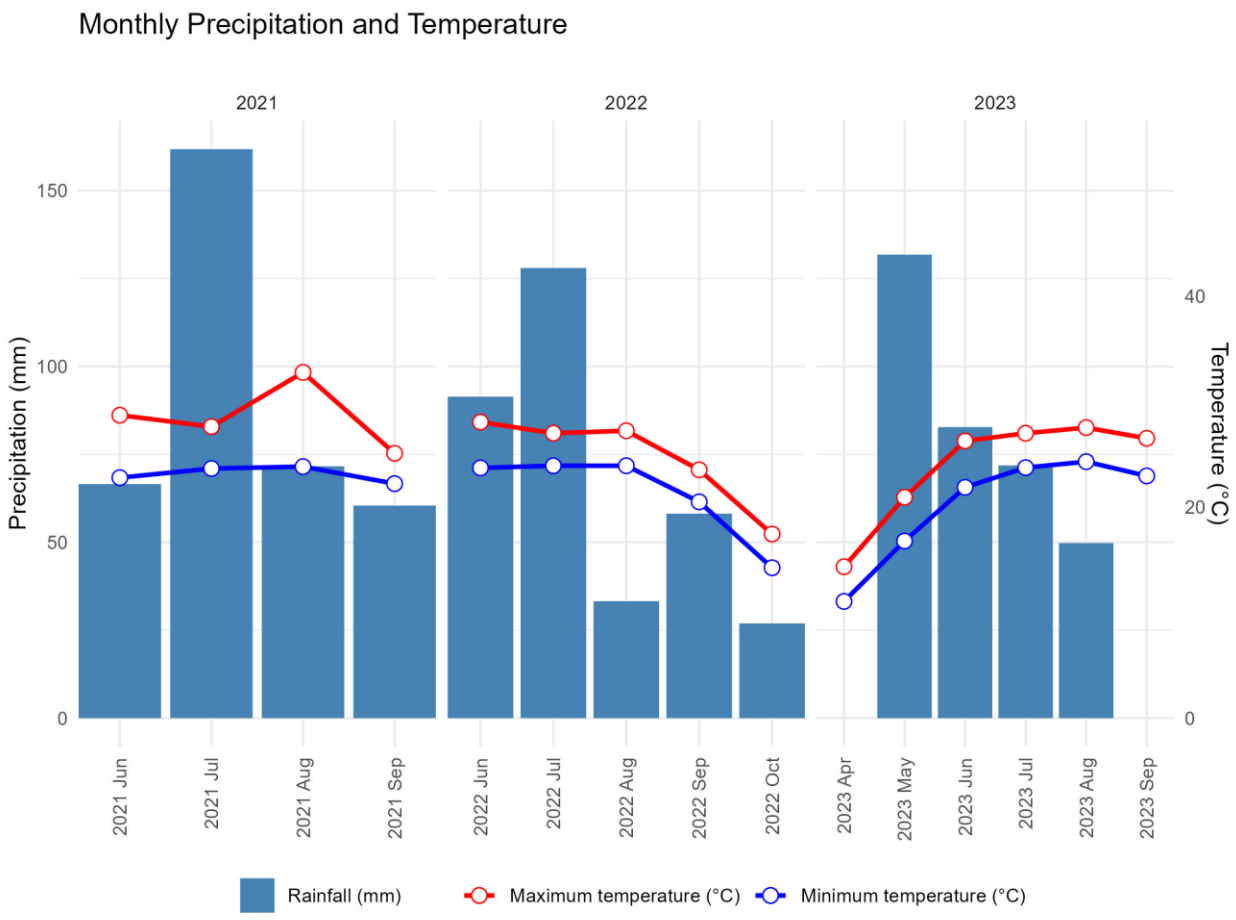

Figure 1. Monthly accumulated rainfall (mm), soil maximum/minimum monthly temperatures during the 2021/22/23 crop season.

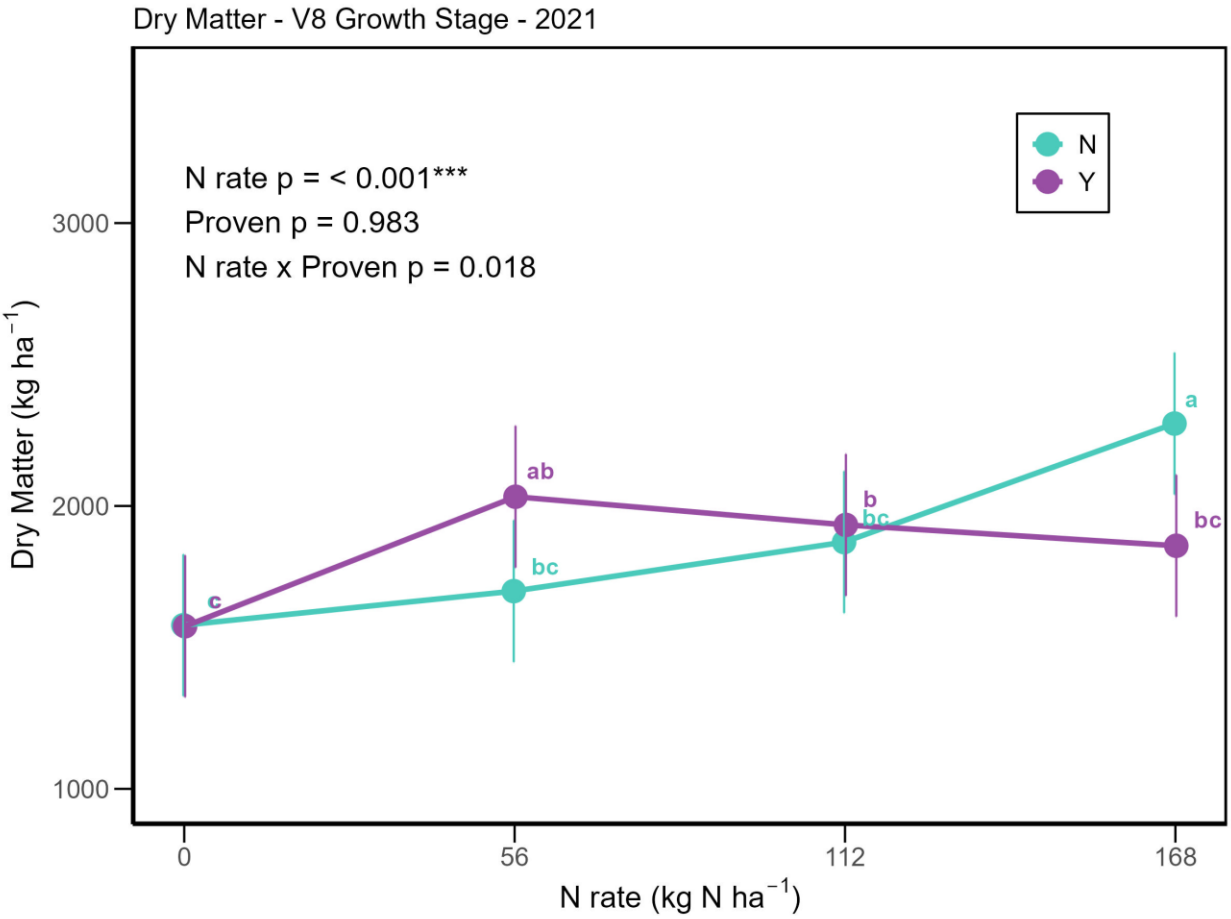

Figure 2. Effect of nitrogen rate and Proven® application ('Y' for Proven®, 'N' for without) on dry matter at the V8 growth stage (2021).

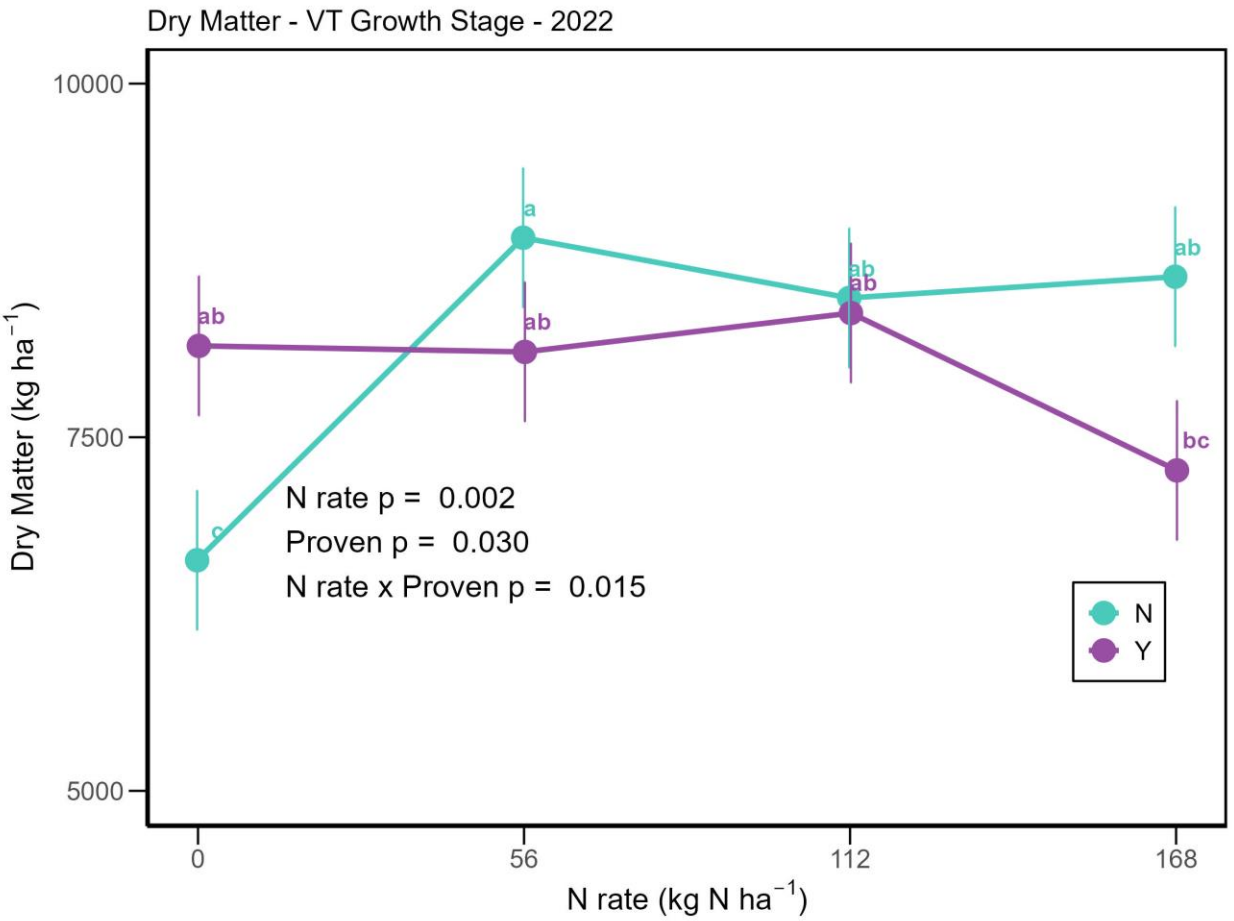

Figure 3. Effect of nitrogen rate and Proven® application ('Y' for Proven®, 'N' for without) on dry matter at the VT growth stage (2022).

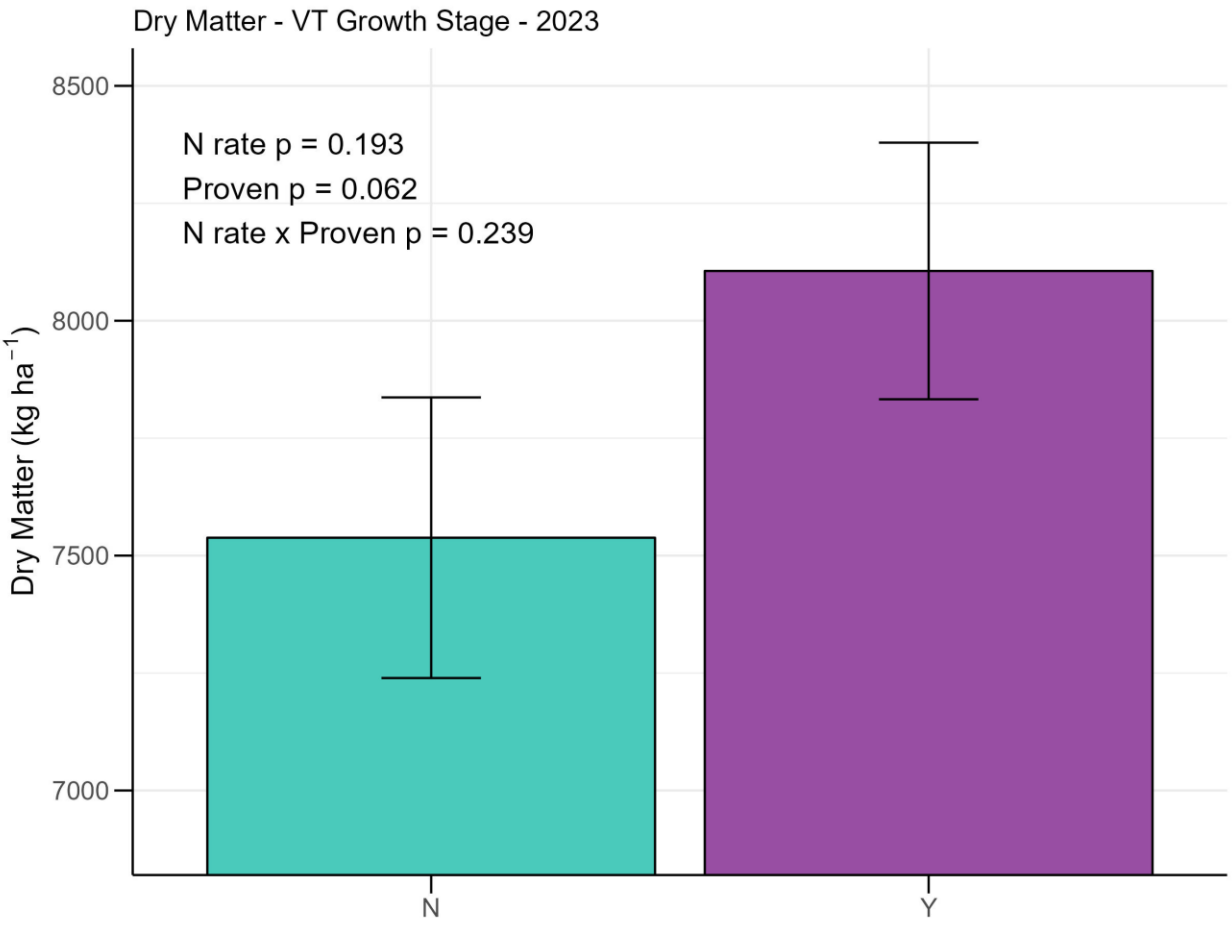

Figure 4. Effect of Proven® ('Y' for Proven®, 'N' for without) on average dry matter accumulation at VT growth stage (2023).

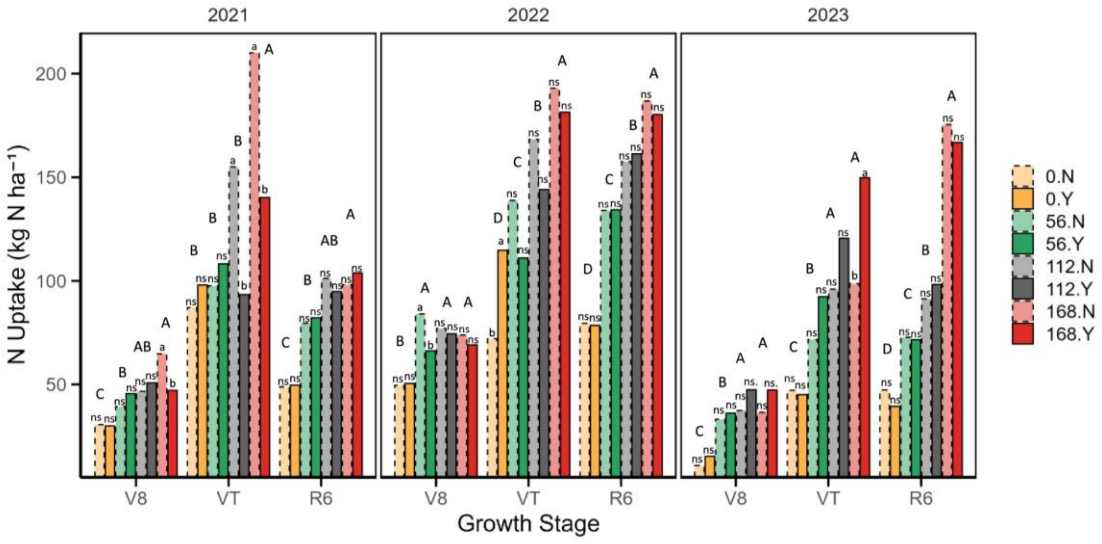

Figure 5. Comparison of nitrogen uptake in corn with and without Proven® at various N rates and growth stages in 2021.

The figure illustrates the nitrogen uptake in corn across three years (2021, 2022, 2023) at three distinct growth stages: V8, VT, and R6. The treatments include the application of Proven® (Y) and no Proven® (N) across varying nitrogen rates (0, 56, 112, 168 kg N ha^{-1}). Capital letters represent the effect of nitrogen (N) rate. Based on pairwise lowercase letters compare Proven® (Y) and no Proven® (N) within each nitrogen rate. The notation **"ns."** Indicates moderate significance, while **"ns"** alone indicates non-significance.

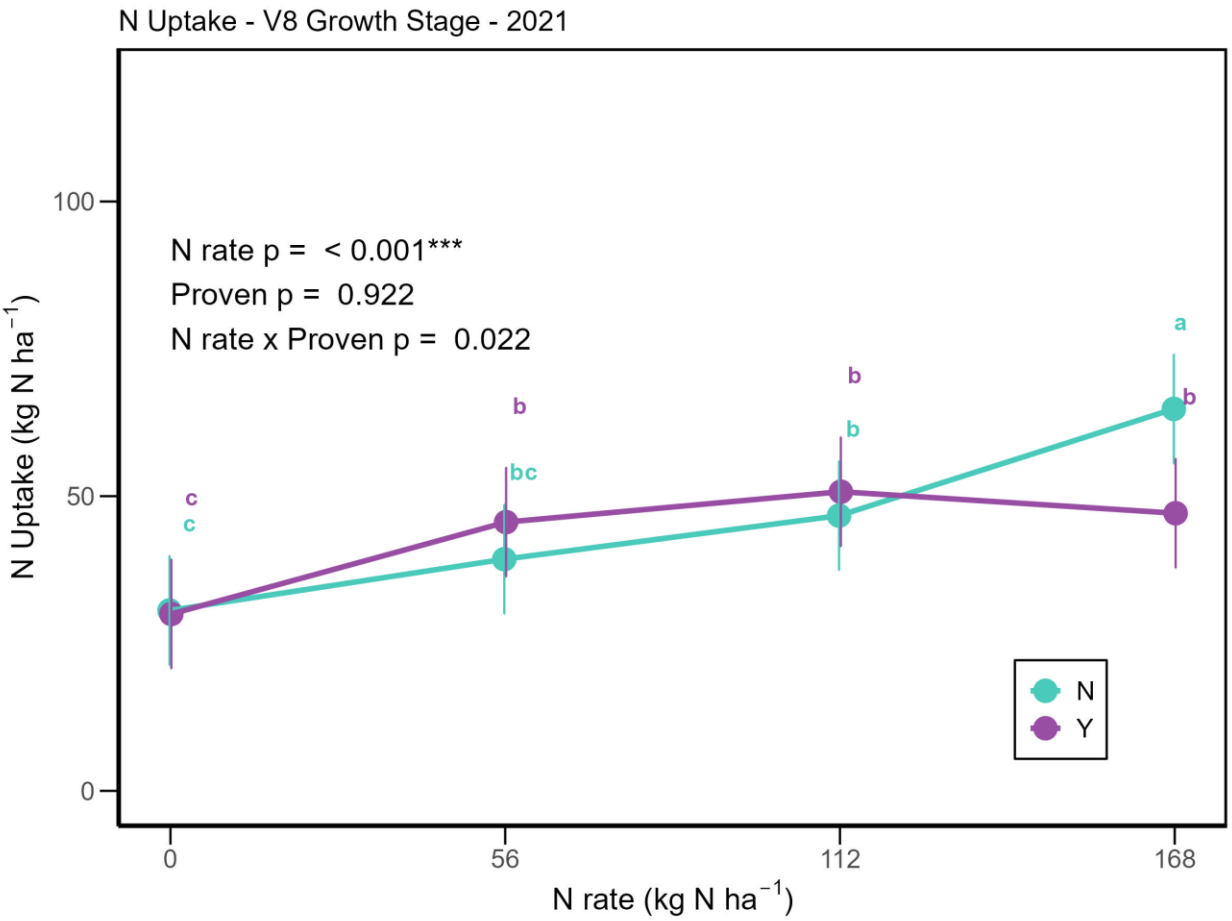

Figure 6. Nitrogen uptake in corn at V8 growth stage with Proven® ('Y') and without Proven® ('N') across different N rates (2021).

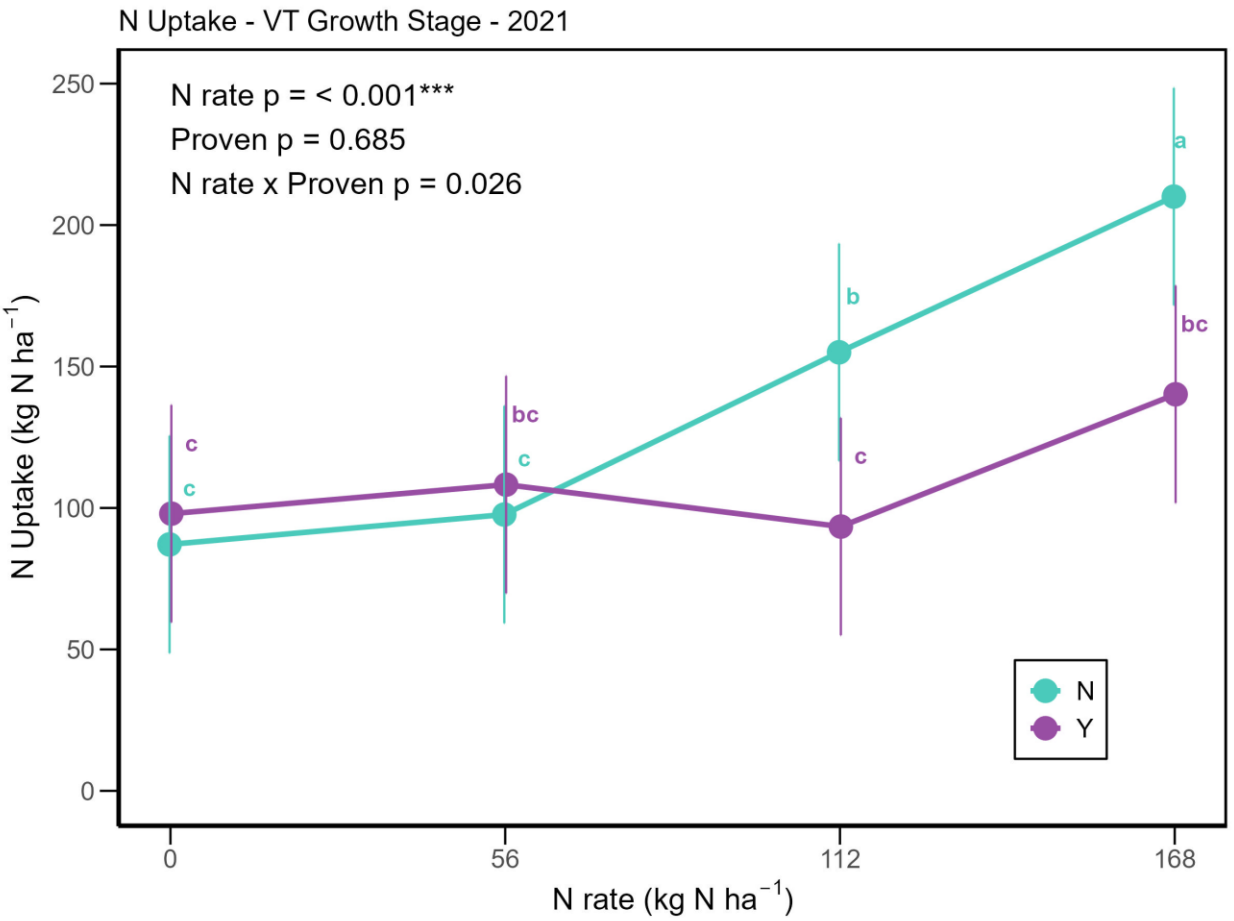

Figure 7. Nitrogen uptake in corn at VT growth stage with Proven® ('Y') and without Proven® ('N') across different N rates (2021).

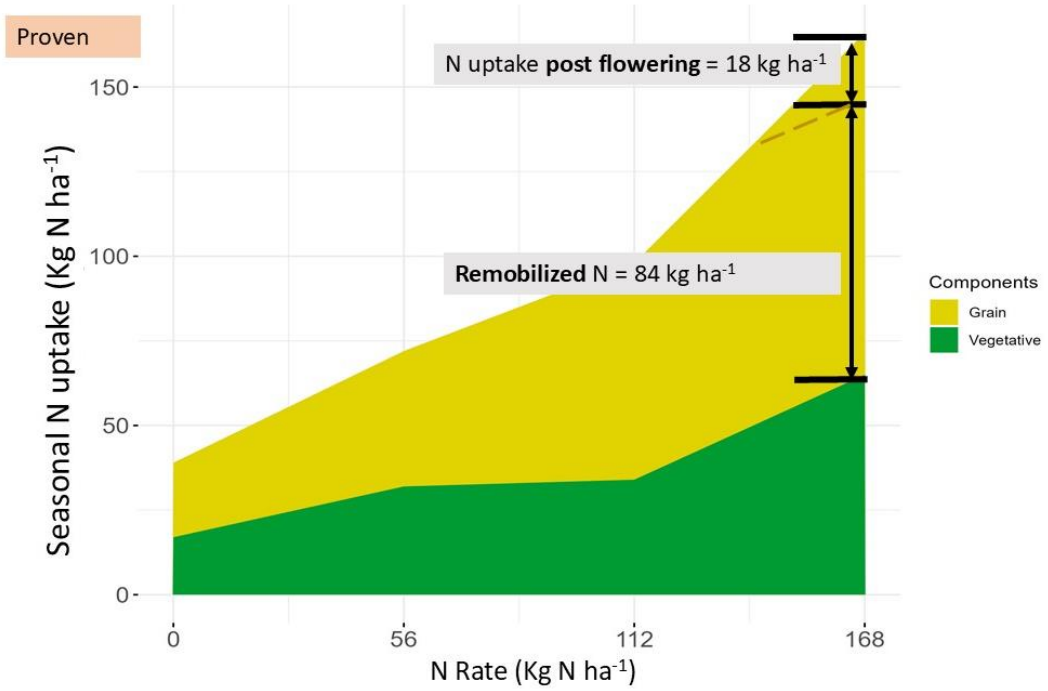

Figure 8. N contribution in the grain from remobilized sources and after VT-R1 (post-flowering) in 2023 with Proven®.

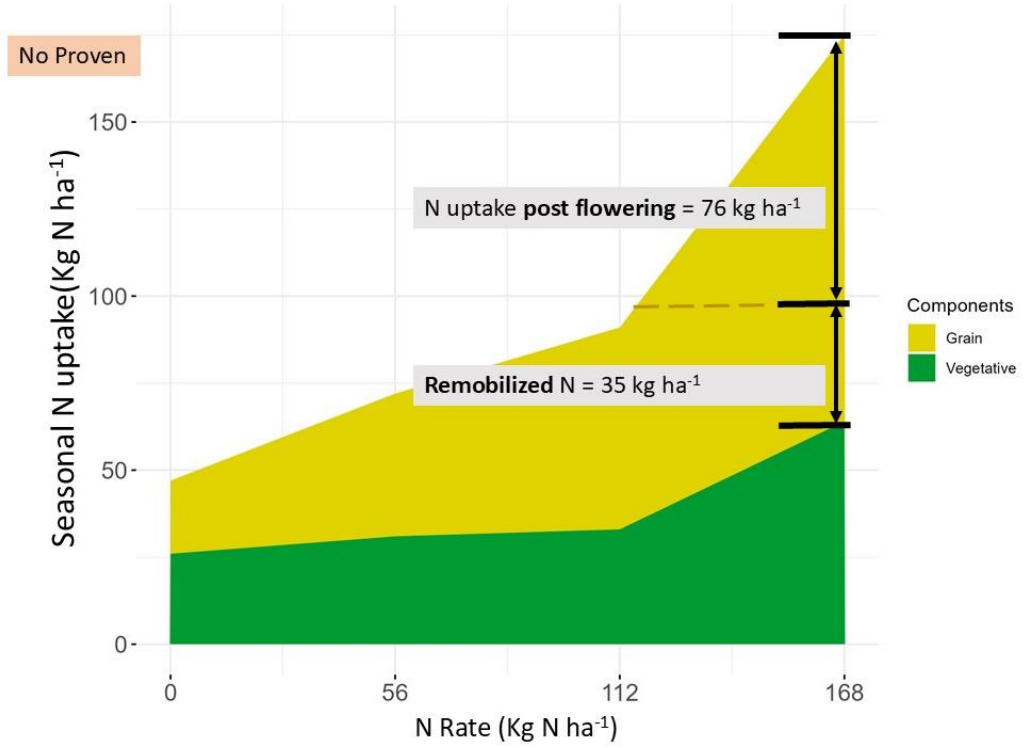

Figure 9. N contribution in the grain from remobilized sources and after VT-R1 (post-flowering) in 2023 without Proven®.

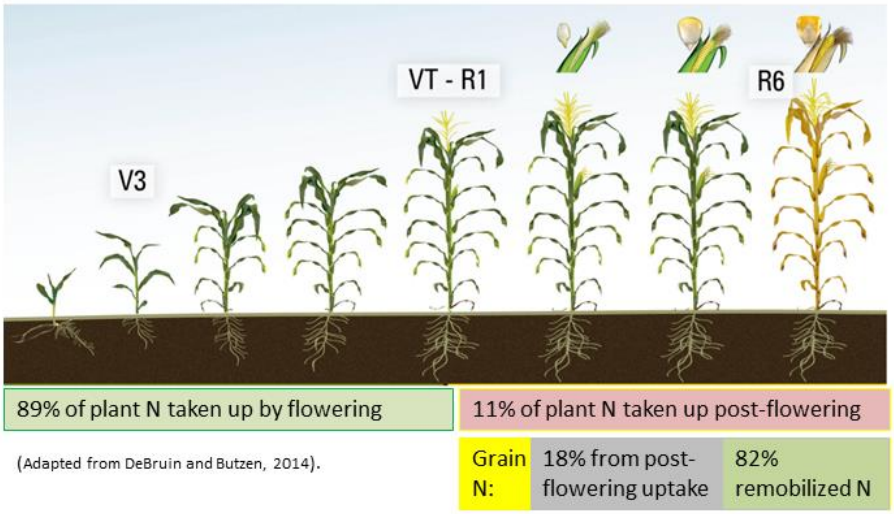

Figure 10. Percentage contribution of nitrogen in grain from remobilized sources and post-flowering uptake (VT-R1 stage) in the 2023 season, assuming a fertilization rate of 168 kg N ha-1 with the use of Proven®. Adapted from DeBruin and Butzen (2014).

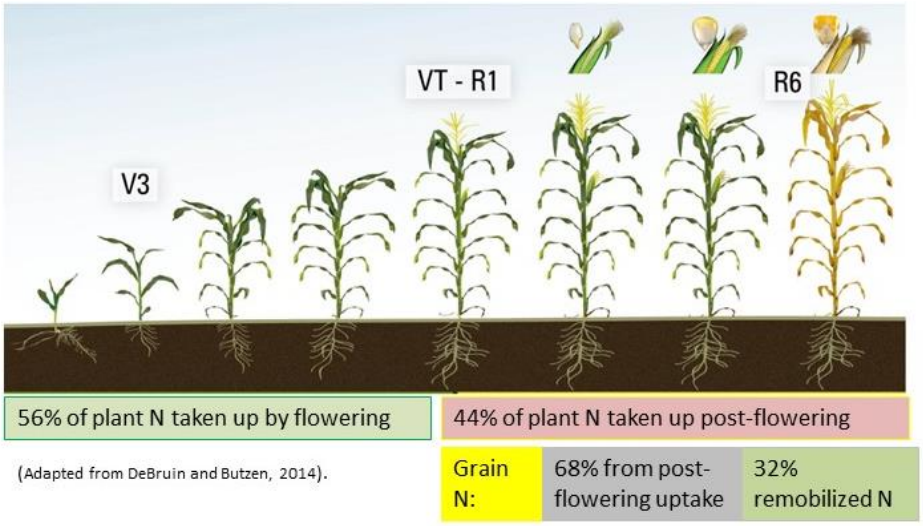

Figure 11. Percentage contribution of nitrogen in grain from remobilized sources and post-flowering uptake (VT-R1 stage) in the 2023 season, assuming a fertilization rate of 168 kg N ha-1 without the use of Proven®. Adapted from DeBruin and Butzen.

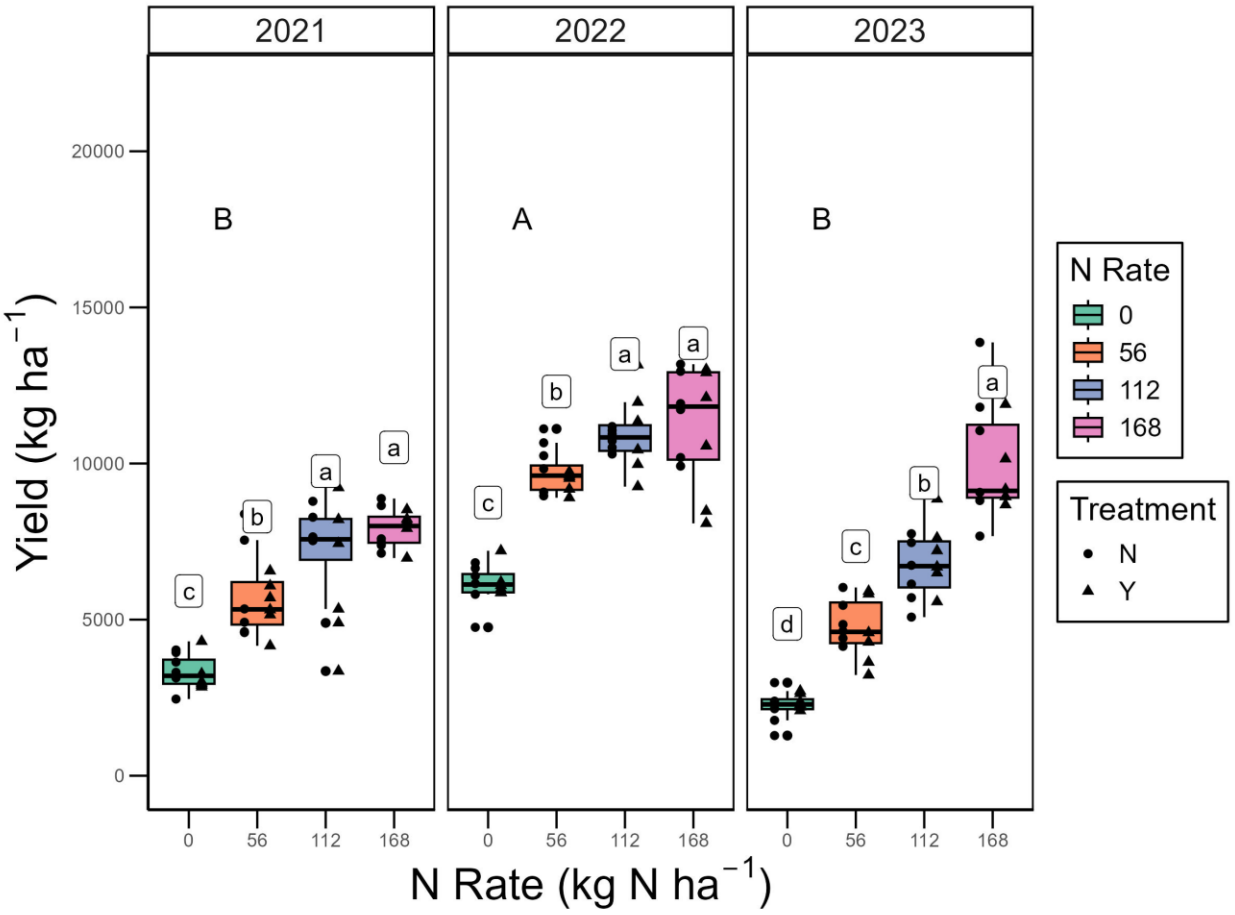

Figure 12. Corn grain yield over three years by nitrogen rate and Proven®.

The effect of yield across the years is represented by the boxplot, categorizing yields by nitrogen rates (0, 56, 112, and 168 kg ha^{-1}), treatment application ('Y' for Proven® and 'N' for without Proven®), and years (2021, 2022, and 2023). Uppercase letters denote statistically significant differences in yield between years, while lowercase letters indicate differences within a year across nitrogen rates.

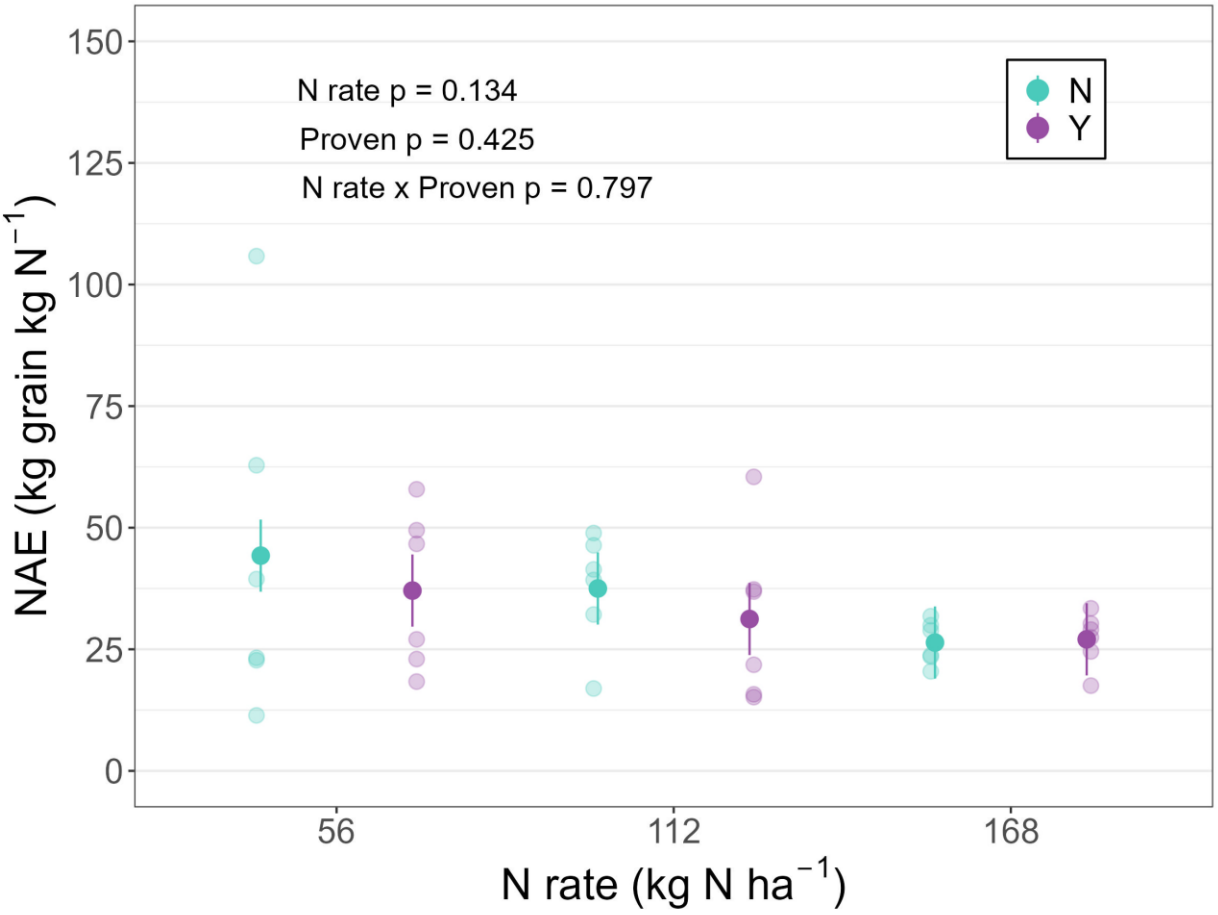

Figure 13. Assessing the Nitrogen Agronomic Efficiency (NAE) in 2021 across different nitrogen (N) rates, with and without the application of Proven®.

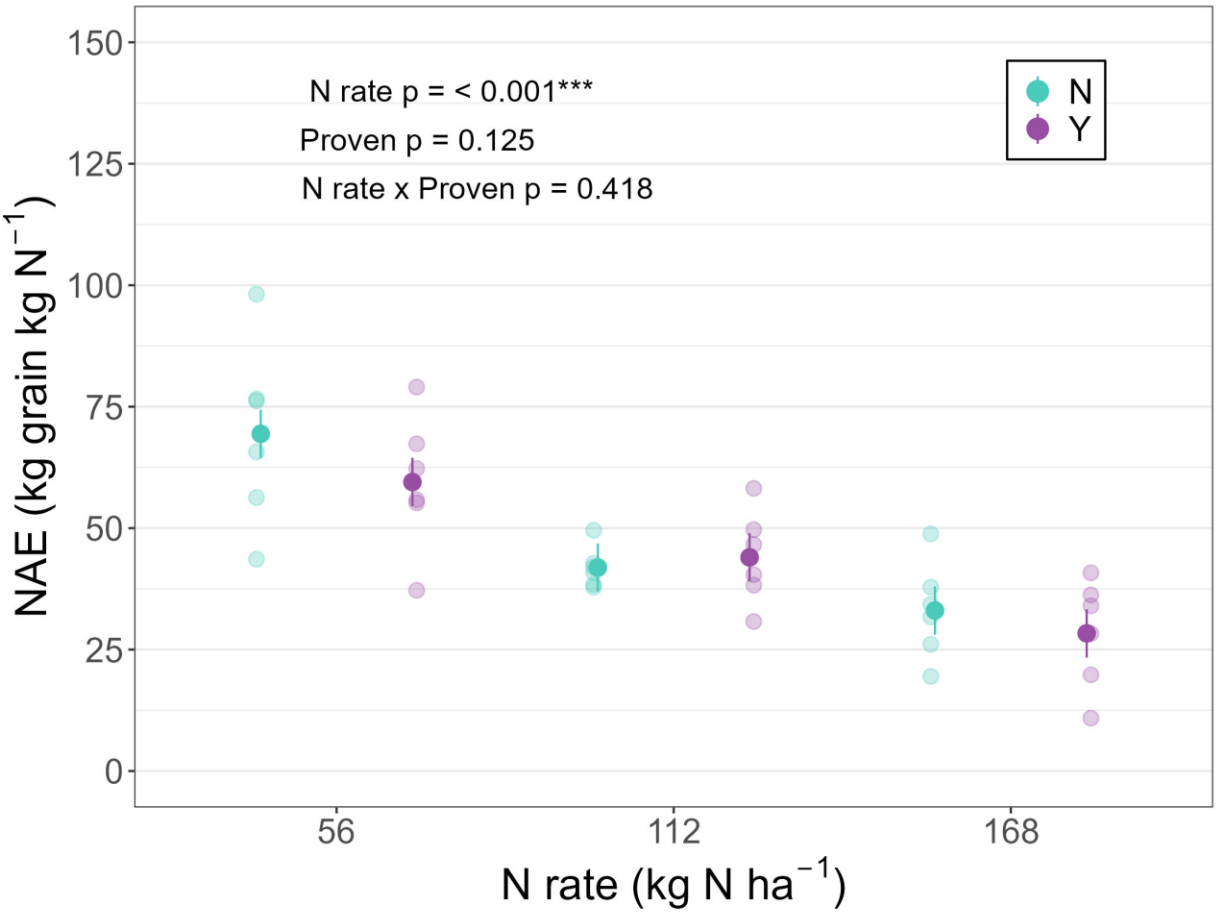

Figure 14. Assessing the Nitrogen Agronomic Efficiency (NAE) in 2022 across different nitrogen (N) rates, with and without the application of Proven®.

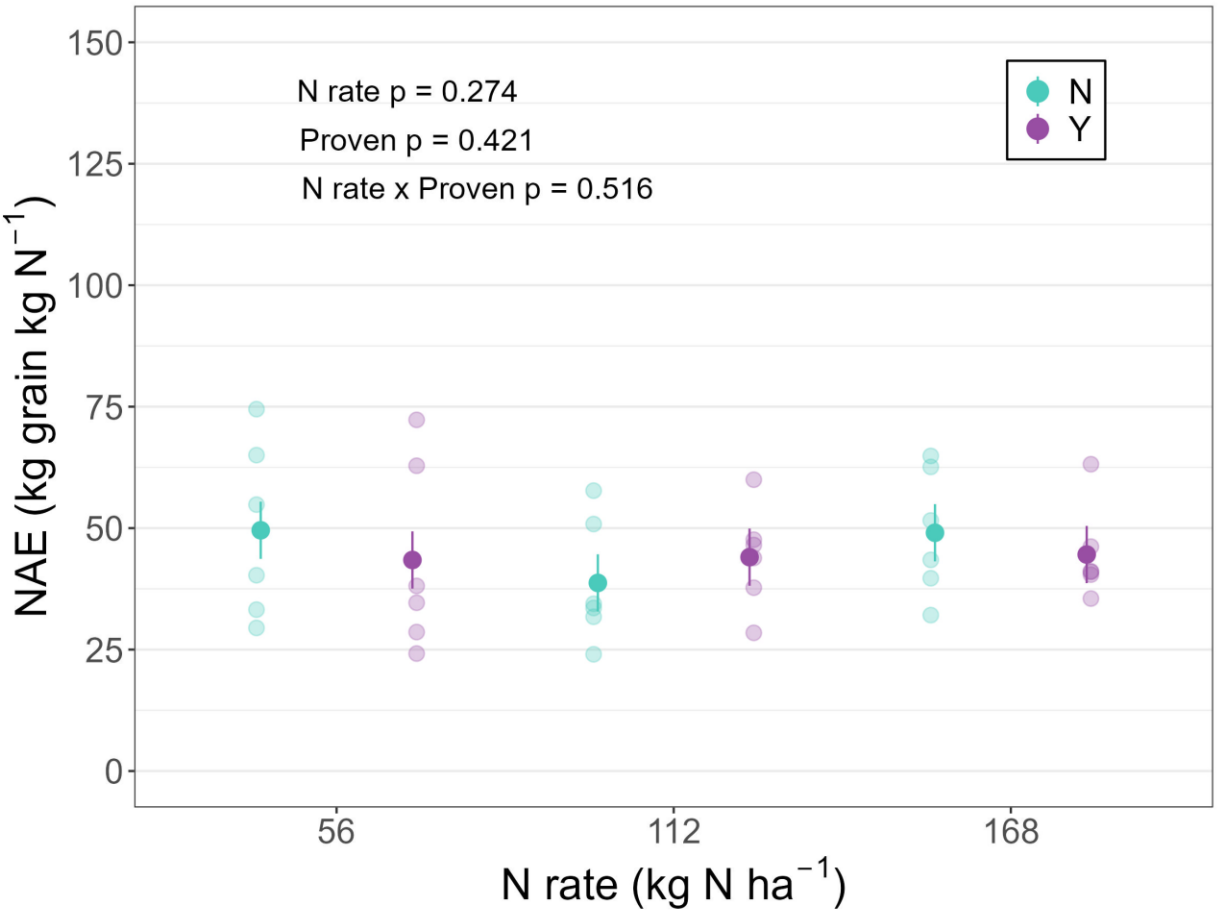

Figure 15. Assessing the Nitrogen Agronomic Efficiency (NAE) in 2023 across different nitrogen (N) rates, with and without the application of Proven®.

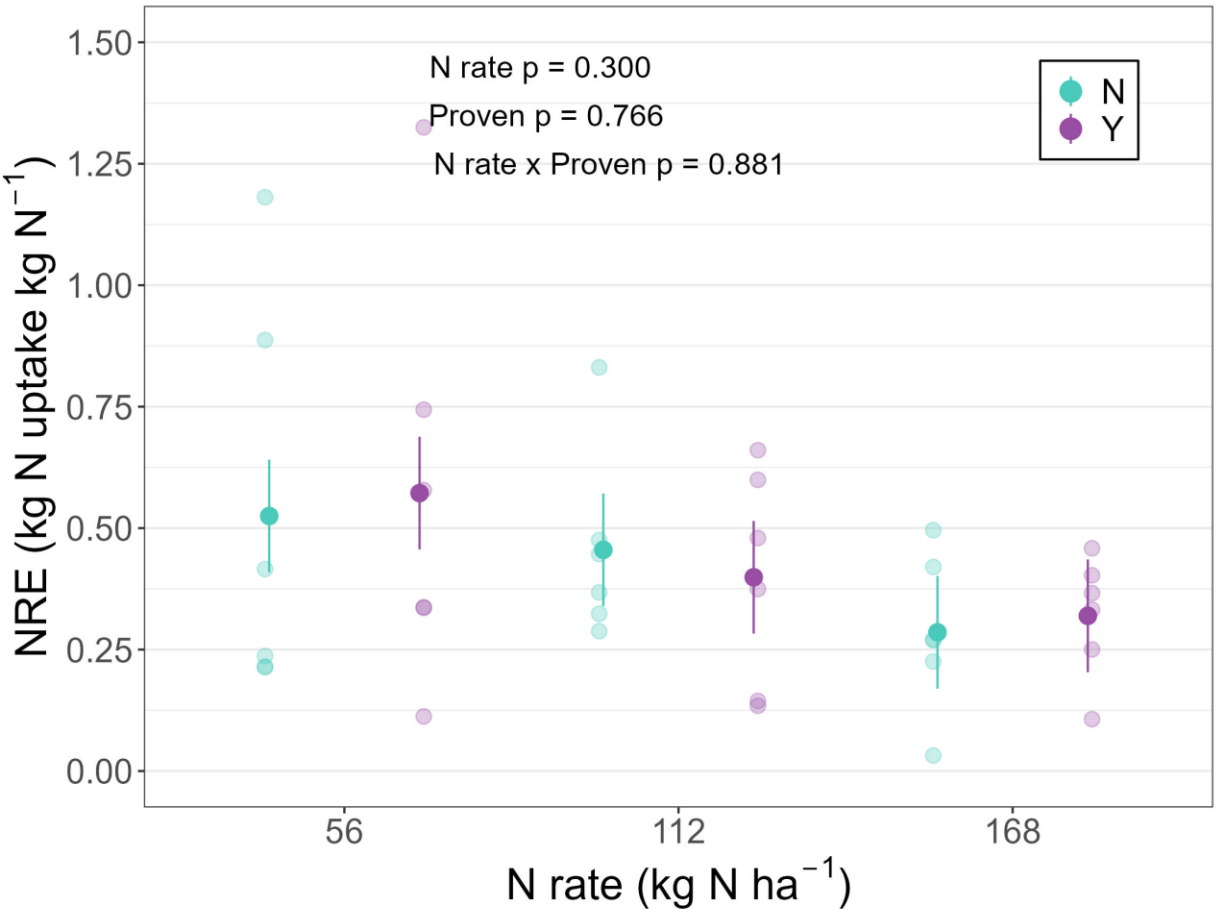

Figure 16. Assessing the Nitrogen Recovery Efficiency (NRE) in 2021 across different nitrogen (N) rates, with and without the application of Proven®.

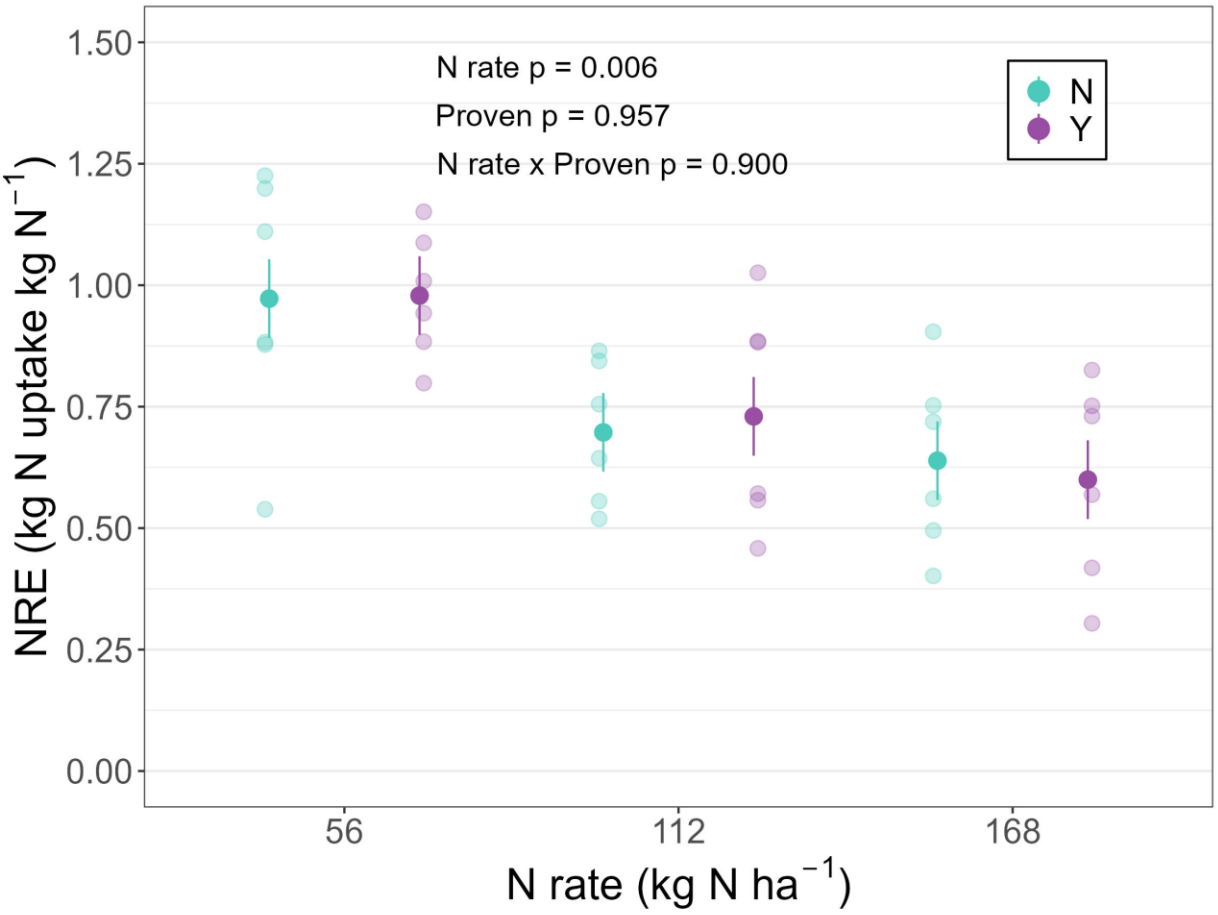

Figure 17. Assessing the Nitrogen Recovery Efficiency (NRE) in 2022 across different nitrogen (N) rates, with and without the application of Proven®.

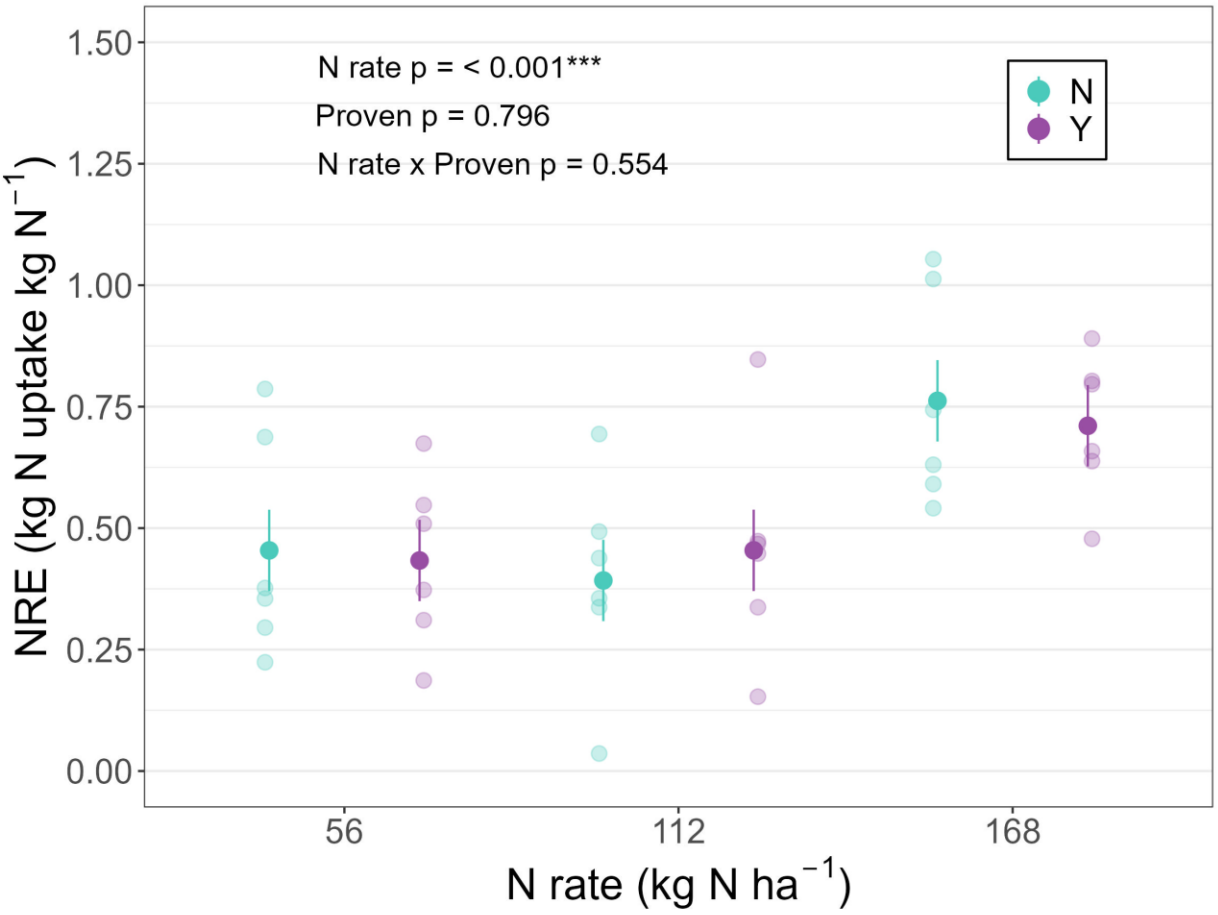

Figure 18. Assessing the Nitrogen Recovery Efficiency (NRE) in 2023 across different nitrogen (N) rates, with and without the application of Proven®.

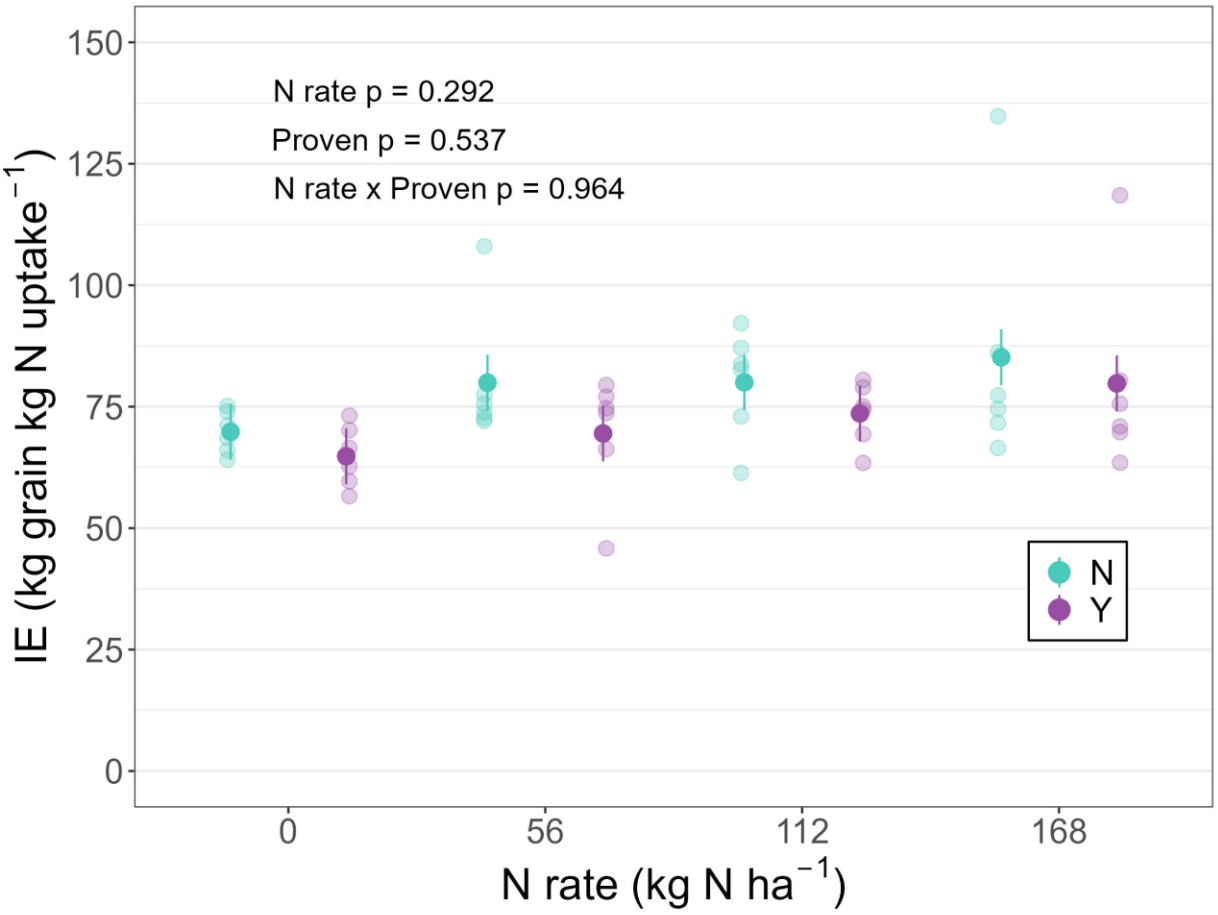

Figure 19. Assessing the Internal Utilization Efficiency (IE) in 2021 across different nitrogen (N) rates, with and without the application of Proven®.

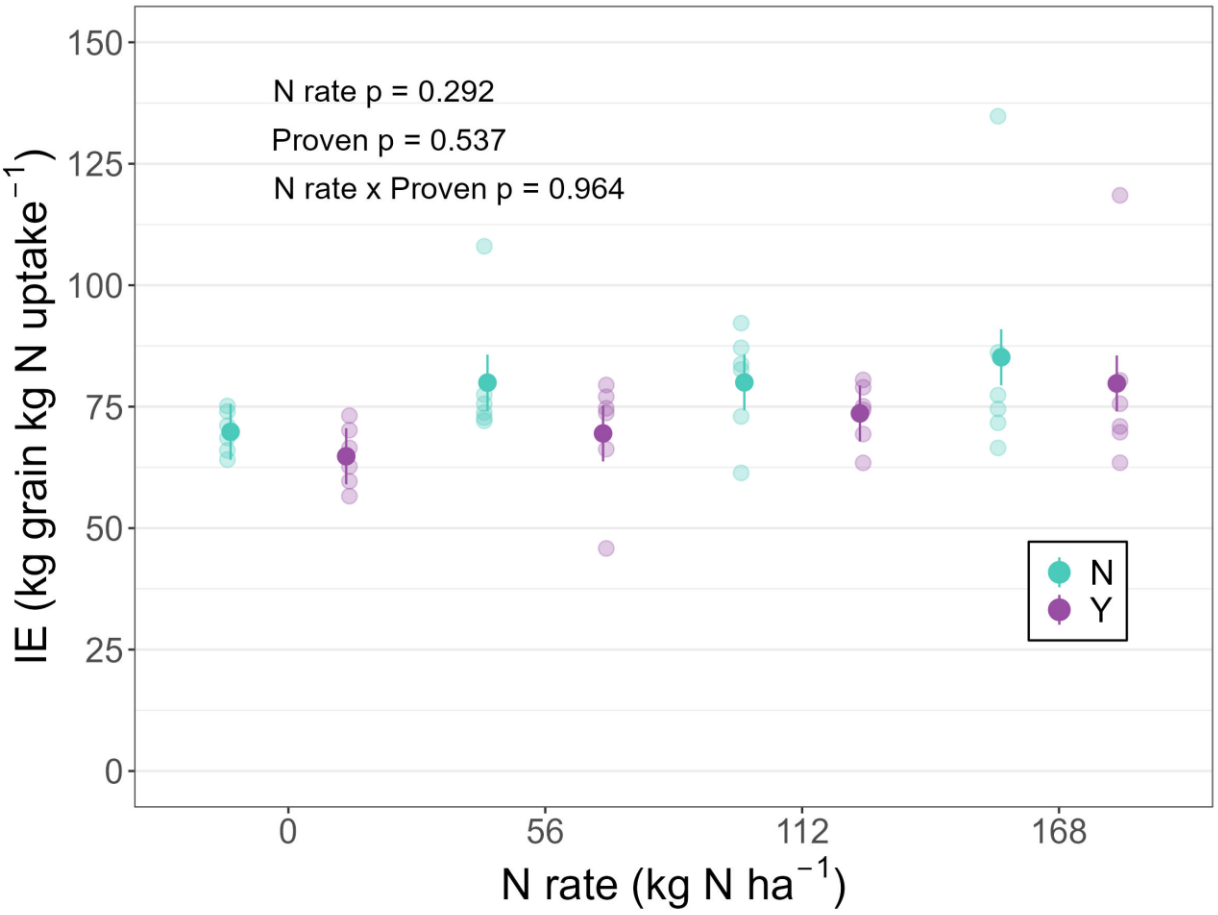

Figure 20. Assessing the Internal Utilization Efficiency (IE) in 2022 across different nitrogen (N) rates, with and without the application of Proven®.

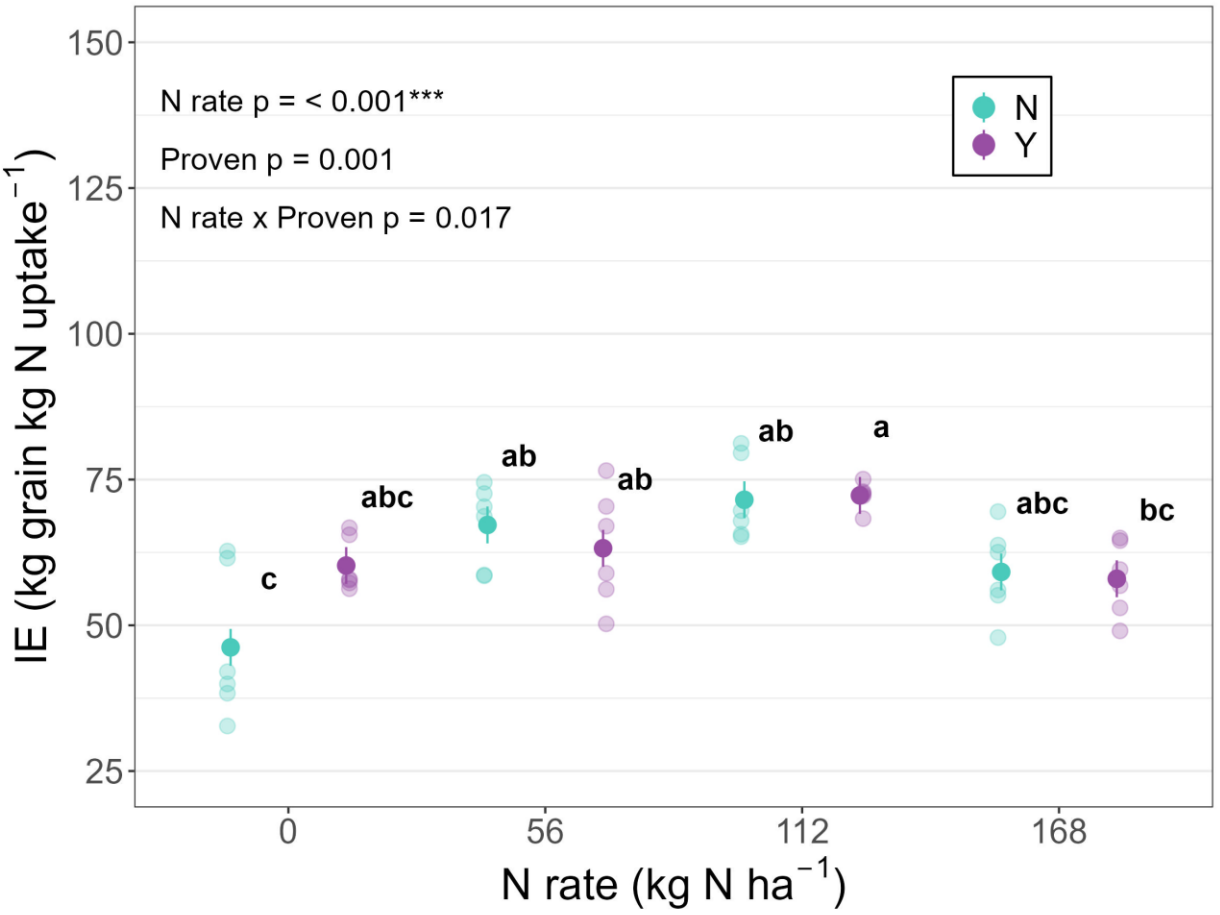

Figure 21. Assessing the Internal Utilization Efficiency (IE) in 2023 across different nitrogen (N) rates, with and without the application of Proven®.

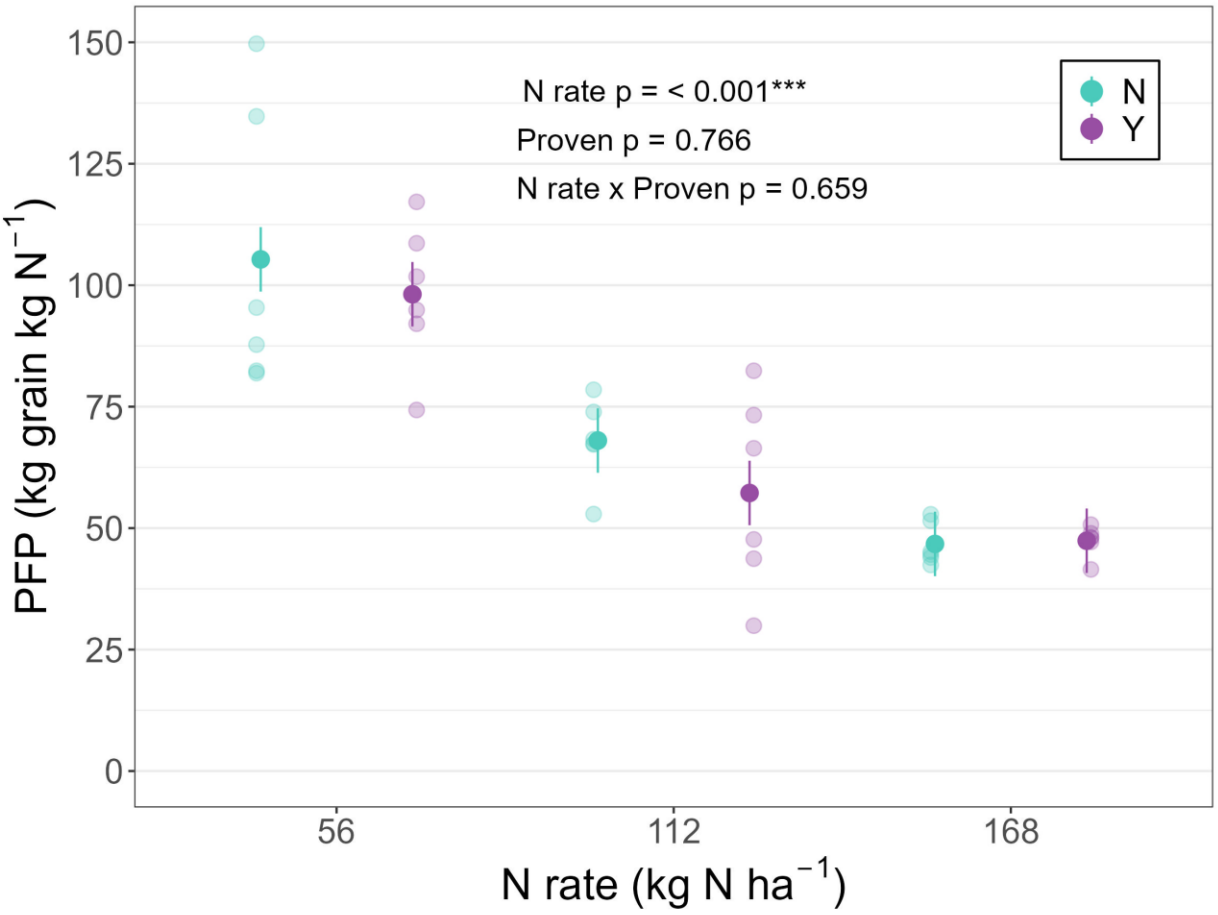

Figure 22. Assessing the Partial Factor Productivity (PFP) in 2021 across different nitrogen (N) rates, with and without the application of Proven®.

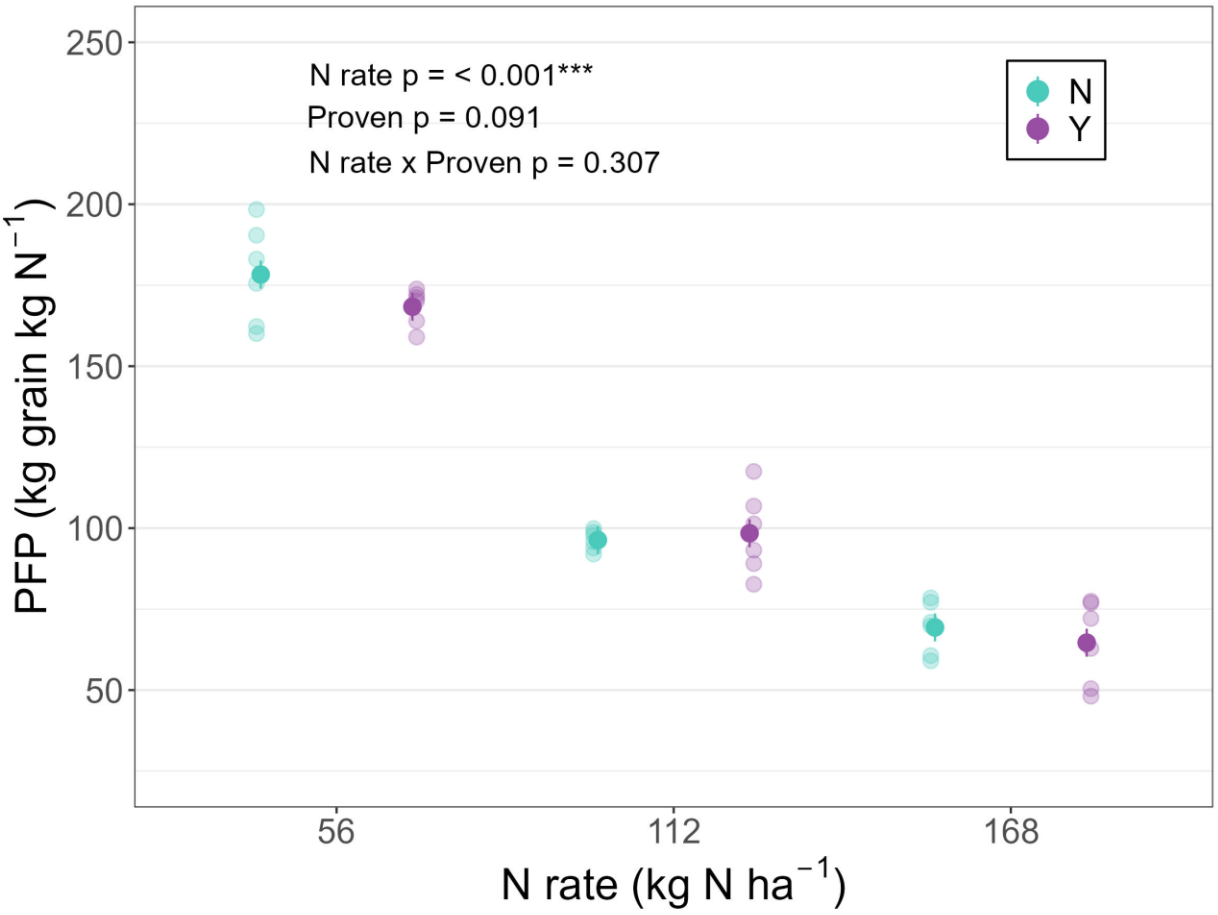

Figure 23. Assessing the Partial Factor Productivity (PFP) in 2022 across different nitrogen (N) rates, with and without the application of Proven®.

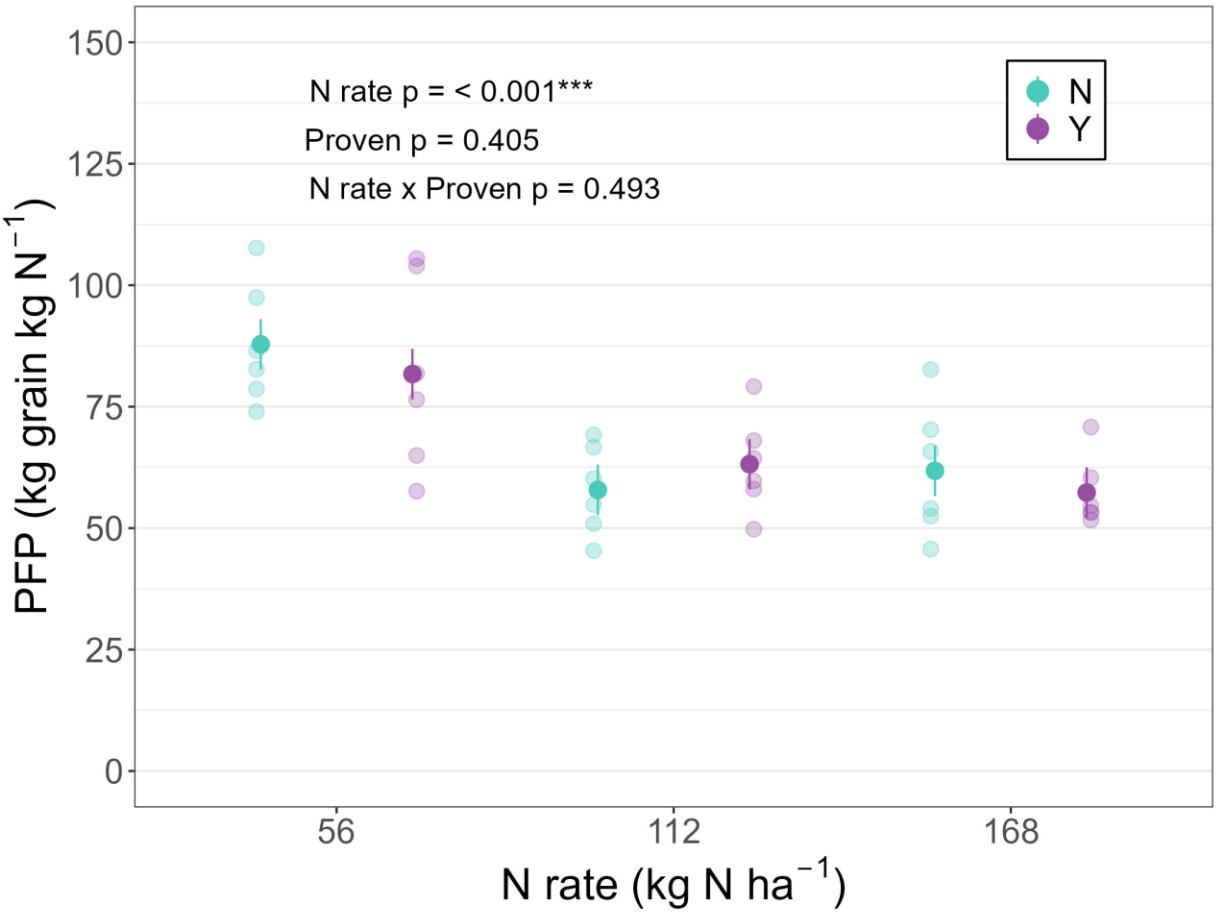

Figure 24. Assessing the Partial Factor Productivity (PFP) in 2023 across different nitrogen (N) rates, with and without the application of Proven®.