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Mid-Season Leaf Glutamine Predicts End-Season Maize Grain Yield and Nitrogen Content in Response to Nitrogen Fertilization under Field Conditions

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Abstract: After uptake in cereal crops, nitrogen (N) is rapidly assimilated into glutamine (Gln) and other amino acids for transport to sinks. Therefore Gln has potential as an improved indicator of soil N availability compared to plant N demand. Gln has primarily been assayed to understand basic plant physiology, rather than to measure plant/soil-N under field conditions. It was hypothesized that leaf Gln at early-to-mid season could report the N application rate and predict end-season grain yield in field-grown maize. A three-year maize field experiment was conducted with N application rates ranging from 30 to 218 kg ha^{-1}. Relative leaf Gln was assayed from leaf disk tissue using a whole-cell biosensor for Gln (*GlnLux*) at the V3-V14 growth stages. SPAD (Soil Plant Analysis Development) and NDVI (Normalized Difference Vegetation Index) measurements were also performed. When sampled at V6 or later, *GlnLux* glutamine output consistently correlated with the N application rate, end-season yield, and grain N content. Yield correlation outperformed GreenSeeker^{TM} NDVI, and was equivalent to SPAD chlorophyll, indicating the potential for yield prediction. Additionally, depleting soil N via overplanting increased *GlnLux* resolution to the earlier V5 stage. The results of the study are discussed in the context of luxury N consumption, leaf N remobilization, senescence, and grain fill. The potential and challenges of leaf Gln and *GlnLux* for the study of crop N physiology, and future N management are also discussed.

Keywords: maize; finger millet; nitrogen; fertilizer; glutamine; biosensor; yield prediction; precision agriculture; plant density; nitrogen use efficiency

1. Introduction

Maize (*Zea mays* L.) is an important cultivated crop and a staple food for billions of people worldwide [1]. There has been a dramatic increase in the production of maize and other cereals such as rice, wheat, barley, sorghum and millet over the last five decades, all of which contribute to global food security [2]. Rising yields can be attributed to advances introduced during the Green Revolution including improved genetics of crop varieties, improved irrigation, and increased pesticide/fertilizer application [3]. Of the fertilizer nutrients, nitrogen (N) is the most abundant in plant biomass and often the most limiting to yield [4,5]. Maize, while an extremely productive crop, has a high N requirement [6] and many farmers fertilize with substantial quantities of synthetic N (e.g., various forms of nitrate and ammonium) to ensure high yields.

In many cropping systems, N fertilizer is typically applied to the field as a single application around the same time as planting [7]. Although N fertilization at the very beginning of the season

is in many scenarios the most convenient, fertilizer nitrogen use efficiency (NUE), defined as the portion of applied N recovered in the aboveground crop biomass, is reduced. N fertilizer (particularly nitrate, NO_3^-), depending on soil type, is susceptible to loss resulting from leaching and denitrification and hence sub-optimal yield and economic return [8]. Substantial N uptake by maize does not occur until plants have fully transitioned from seed to soil N reserves [9]. Exponential biomass accumulation is initiated 4–6 weeks after planting at approximately the V6 stage (a growth stage defined as when six leaves have fully emerged from the plant) and continues through the reproductive growth stages (beginning at R1) [9].

Considerable variability in N supply, loss and demand can exist within a single field and across years [10], dictated by factors including soil type, temperature, precipitation, microbial activity, and planting density [8]. The combined effect can result in substantial fertilizer N rate heterogeneity [10,11]. To better account for this variability and to match N fertilizer application with maize N requirement, growers are advised to provide N mid-season (sidedressing, topdressing) in addition to, or as a replacement for, the starter fertilizer which is applied earlier at the beginning of the season [7]. When fertilizer applications are synchronized with the needs of the plant they can result in higher fertilizer NUE [12]. Mid-season application rates are sometimes determined in a site-specific manner based on measurable indicators of plant N health (e.g., chlorophyll concentration) or soil N content [13–15]. Commonly used diagnostic tests to measure mid-season plant N health and guide the N application rate include the Soil Plant Analysis Development (SPAD) meter (Konika Minolta, Tokyo, Japan) which provides a measurement of foliar chlorophyll concentration, and the Normalized Difference Vegetation Index (NDVI) which estimates plant vigour via detection of the near-infrared spectral region reflected by plant leaves. NDVI can be measured by several devices including the GreenSeeker™ (Trimble, Westminster, CO, USA) handheld crop sensor [16,17]. These diagnostic tools measure N-dependent metrics such as chlorophyll and vegetative area. Aside from its requirement for photosynthesis, chlorophyll is also used by plants as a form of long-term N storage [18]. This factor may contribute to SPAD and NDVI exhibiting limited response to different N application rates, soil N test values, and leaf N concentrations, with plateaus observed at high N values [19–25]. Furthermore, measurements dependent upon chlorophyll (SPAD) and vegetative area (NDVI) can have problems especially early in the season—the correlation between measures of leaf N health and different starter N fertilization rates often does not occur until V6 or later [26,27]. Initial studies indicated that early-season SPAD measurements correlate very weakly with end-season yield, with coefficients as low as 0.22 even at V7 [28–30]. Later research identified the correct leaf and position for SPAD measurement, which increased its yield-predictive value. However, low correlation coefficients are still frequently reported, for example 0.48 at V6 [31,32]. The correlation between NDVI and yield is also often low and variable, especially early in the season (V6) [26,27,33]. Therefore it would be of value to develop diagnostic tools that can detect plant N limitation earlier and that better correlate with end-season grain yield and N content, for diagnosis of crop N status in the field. If such a measure were combined with dynamic models that integrate cereal crop growth, crop history, climate, soil, and agronomic factors [34–42], it may allow for better determination of N requirement and therefore selection of a mid-season N application rate.

After soil N is absorbed by cereal crop roots, it is assimilated into a pool of free amino acids for the production of proteins and other macromolecules [43]. In maize, glutamine (Gln) is one of the most abundant assimilatory amino acids and a major N transport molecule, displaying marked increases in foliar tissue immediately after N application to roots [44,45]. The N assimilatory and Gln translocation ability of a plant contribute to NUE. Therefore free Gln in maize tissue has potential to serve as an exquisite and responsive indicator of the ratio between N supply and demand. Gln has primarily been measured to understand basic plant physiology [45–48], rather than as a measurement of plant N status under field conditions.

It can be assumed that there are three broad scenarios concerning plant N health in the field, with different implications for Gln. Where there is insufficient soil N supply to meet plant N demand,

the amount of Gln transported will consequentially be low. Alternatively, if the demand is met by sufficient soil N, then the amount of Gln transported will be comparatively high. A final situation is one in which the N available to the plant from the soil exceeds demand, and the consequent concentration of translocated Gln may accumulate [49–51]. Free Gln may act as an endogenous signal of whether the plant is N-limited, sufficient, or in a luxury situation—Gln in plant tissue can impose negative regulation on N transporters in plant roots [52].

We previously reported a whole-cell biosensor for Gln called *GlnLux*, consisting of a strain of *Escherichia coli* bacteria, auxotrophic for Gln, that was genetically engineered to emit photons of light as it grew and multiplied proportional to the concentration of Gln supplied, including from leaf extracts [53]. Under controlled greenhouse conditions, *GlnLux* was able to detect rapid increases of Gln (*GlnLux* glutamine) in maize leaf tissue after ammonium nitrate was applied to seedlings, and its low cost and low tissue requirement (a small leaf disk from the midrib) provided detailed temporal and spatial mapping of Gln dynamics [54].

Here, it was tested whether Gln in maize leaves, as measured with *GlnLux*, can be used as a diagnostic of soil N availability as inferred from the correlation with the N application rate. Further, it was tested whether the early-to-mid season leaf Gln status correlated with end-season yield traits under N-limiting field conditions. The yield correlations were compared to early-to-mid season SPAD chlorophyll, and GreenSeeker^{TM} NDVI. A field experiment was conducted over three years in which N was applied shortly after planting at rates ranging from 30 to 218 kg ha^{-1}. *GlnLux* glutamine was examined in maize foliar tissue at four different stages (V3, V6, V12, and V14).

2. Results

2.1. Correlation of Leaf GlnLux Glutamine and Nitrogen Application Rate

Maize was grown in a field across three years (see Supplementary Materials Figure S1 for weather conditions, adapted from data obtained in [55]) with different levels of nitrogen (N) fertilizer applied shortly after planting. Before N was applied, it was determined by multiple means comparison that none of the N treatment plots differed in either ammonium or nitrate content in any of the three years (data not shown). Midrib disks from young leaves were analyzed with the *GlnLux* biosensor for relative glutamine (Gln) content, the output of which is referred to as *GlnLux* glutamine (Supplementary Materials Figure S2). In all three years, *GlnLux* glutamine correlated with N application rate when leaves were sampled later in vegetative growth (V12, V14), and as early as the V6 growth stage in 2015 and 2016 (Table 1; Figure 1A–D). Significant correlation was not observed in any of the three years at V3, the earliest growth stage sampled.

Table 1. Pearson correlation between maize *GlnLux* glutamine and end-season measurements [z].

Growth Stage	V3			V6			V12			V14		
Year	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**
N rate	0.08	−0.19	−0.06	−0.02	0.70 ***	0.74 ***	0.59 **	0.47 *	0.72 ***	0.87 ****	0.90 ****	0.87 ****
Grain yield [y]	−0.04	−0.27	−0.16	0.02	0.78 ****	0.68 ***	0.74 ***	0.56 **	0.77 ****	0.83 ****	0.82 ****	0.90 ****
Ear dry wt	0.12	−0.17	−0.17	−0.3	0.60 **	0.76 ***	0.65 **	0.47 *	0.62 **	0.66 **	0.62 **	0.84 ****
Grain dry wt	0.11	−0.18	−0.17	−0.23	0.59 **	0.76 ***	0.65 **	0.46 *	0.61 **	0.66 **	0.62 **	0.84 ****
Stover dry wt	0.25	−0.18	−0.14	−0.39	0.54 *	0.62 **	0.56 *	0.43	0.42	0.48 *	0.54 *	0.62 **
Grain N%	0.06	0.19	0.07	−0.22	0.27	0.69 ***	0.58 **	0.36	0.64 **	0.67 **	0.21	0.68 ***
Grain NC	−0.01	−0.2	−0.1	−0.07	0.76 ****	0.74****	0.71 ***	0.59 **	0.77 ****	0.82 ****	0.77 ****	0.88 ****
Stover N%	−0.45	0.4	0.25	0.23	0.06	0.33	0.42	0.03	0.60 **	0.4	0.24	0.58 **
Stover NC	−0.17	0.26	0.05	−0.1	0.54 *	0.47 *	0.64 **	0.39	0.71 **	0.61 **	0.66 **	0.73 ***
HI	−0.01	−0.28	−0.15	−0.21	0.50 *	0.64 **	0.68 **	0.33	0.62 **	0.71 ***	0.58 **	0.84 ****
NHI	−0.04	−0.4	−0.22	0.13	0.25	0.75 ***	0.65 **	0.21	0.55 *	0.67 **	0.12	0.76 ***

[z] All measurements were performed across three years and four replications. Variety was Dekalb DKC39-97RIB; [y] Grain yield at 15.5% moisture content; One, two, three, and four asterisks represent significant Pearson correlation at $p < 0.05$, 0.01, 0.001, and 0.0001 respectively; HI: harvest index; N: nitrogen; NHI: nitrogen harvest index; wt: weight; NC: nitrogen content.

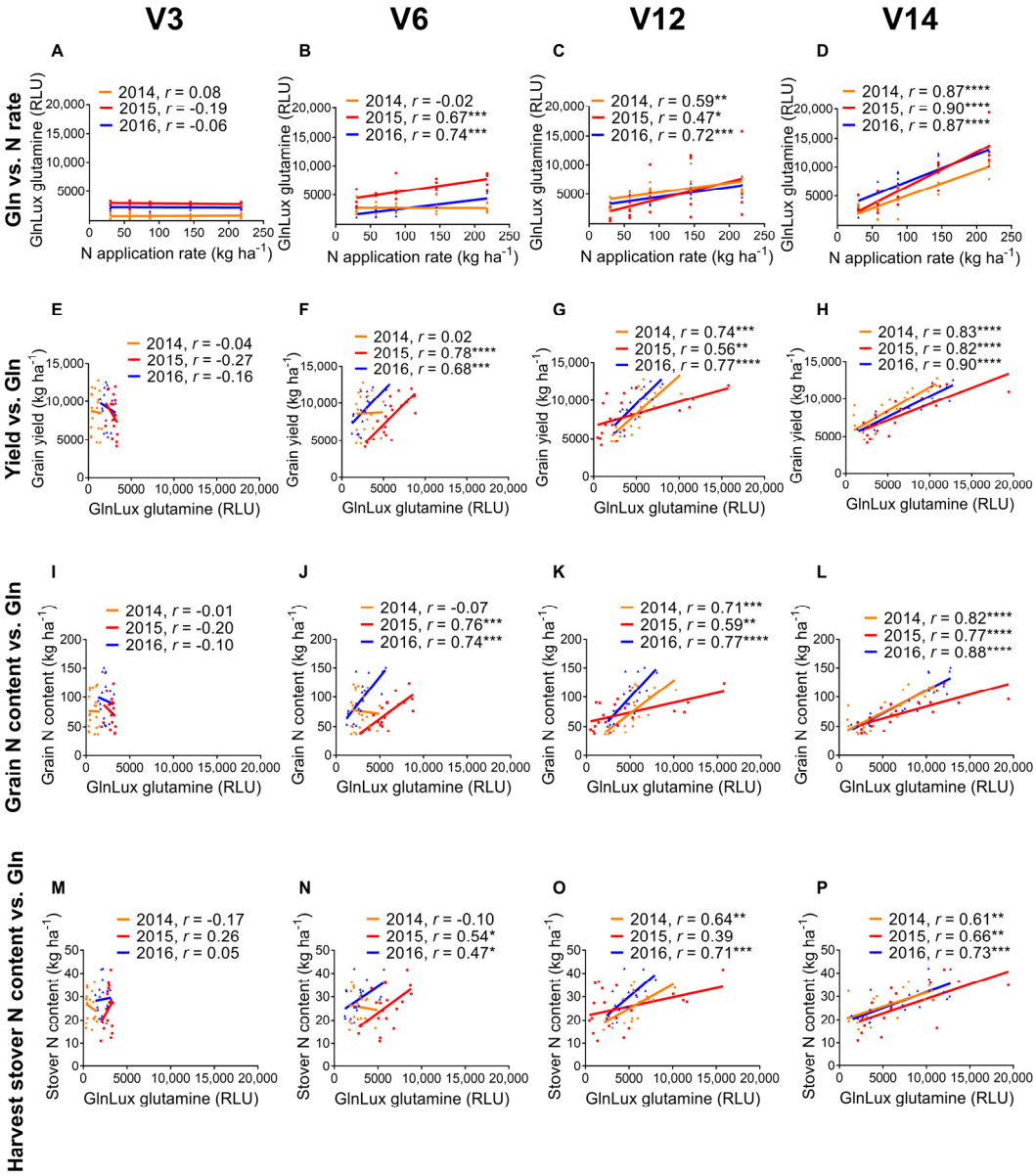

Figure 1. *GlnLux* glutamine values from maize leaf tissue displays increasing correlation with end-season measurements as vegetative growth stage (V3 to V14) progresses. Pearson correlation of *GlnLux* glutamine levels was performed against (**A**–**D**) N application rate; (**E**–**H**) grain yield; (**I**–**L**) grain N content and (**M**–**P**) end-season stover N content. One, two, three, and four asterisks beside correlation coefficients of each year represent significance at $p < 0.05$, 0.01, 0.001, and 0.0001 respectively. RLU, relative light units intercepted by the luminometer in a one second interval.

2.2. GlnLux Glutamine Correlation with End-Season Field Measurements

Relationships were tested between various end-season traits and leaf *GlnLux* glutamine sampled at different vegetative growth stages (V3, V6, V12, and V14) (Table 1). For grain yield, ear dry weight, grain dry weight, and total grain N content, moderate to strong Pearson correlations were consistently observed between leaf *GlnLux* glutamine throughout vegetative growth after V12 in all growing years, and after V6 in 2015 and 2016 (Table 1; Figure 1E–L). Significant correlations were also observed, although more variably, between *GlnLux* glutamine and grain and stover total N percentage, stover dry weight, total stover N content, harvest index, and nitrogen harvest index (NHI, ratio between N content

in the grain and of the total above-ground tissues of the plant) (Table 1; Figure 1M–P). Overall, stronger correlations were most likely to occur later in the season (after V6) (Table 1; Figure 1E–P).

2.3. Comparison of the Yield-Predictive Potential of Vegetative GlnLux Glutamine, SPAD Chlorophyll, and GreenSeeker™ NDVI

The yield-predictive potential of *GlnLux* glutamine (Figure 2A–C) was compared to commercial indicators of in-season N health, specifically SPAD chlorophyll (Figure 2D–F) and GreenSeeker™ NDVI (Figure 2G–I). Leaf measurements at V6 and V12 were plotted against end-season grain yield adjusted to 15.5% moisture. *GlnLux* glutamine showed similar yield correlation values as SPAD chlorophyll in 2015 and 2016, but showed a stronger correlation in 2014 (Figure 2A–F). Of the three measurements tested, GreenSeeker™ NDVI displayed the least correlation with end-season yield—no relationship at V6 was observed. Only V12 measurements correlated with yield in 2015 and 2016, with no correlations observed in 2014 at either growth stage (Figure 2G–I).

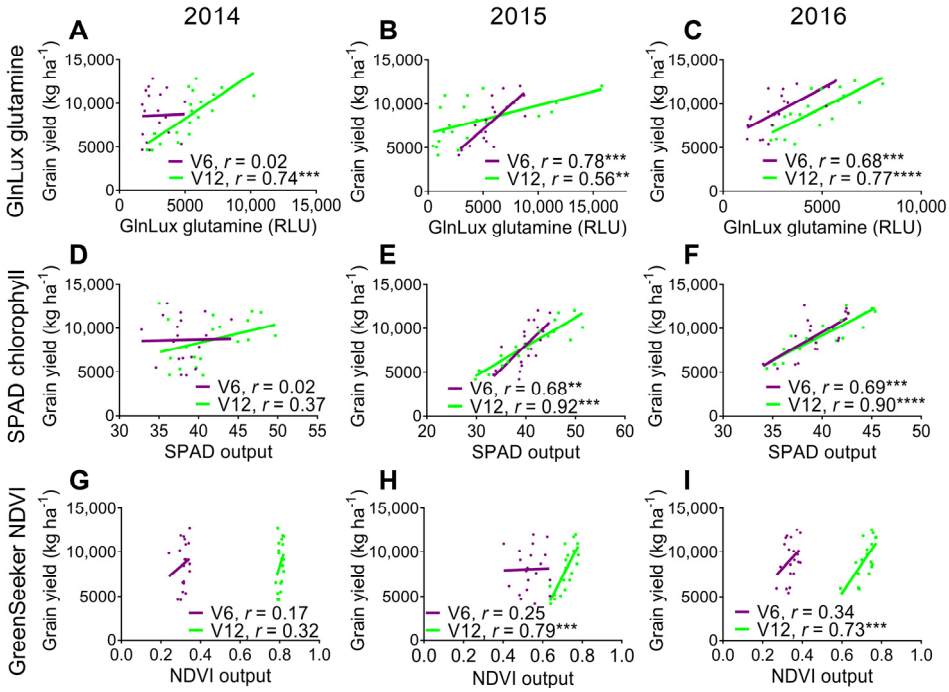

Figure 2. Correlation of end-season maize grain yield (kg ha^{-1}) and (**A**–**C**) *GlnLux* glutamine; (**D**–**F**) SPAD; and (**G**–**I**) NDVI output. Maize was provided with N application rates of 30, 58, 87, 145, or 218 kg ha^{-1}. All measurements were recorded at the V6 and V12 growth stages in 2014, 2015, and 2016. Asterisks represent significant Pearson correlations at $p < 0.05$, <0.01, <0.001, and <0.0001. RLU, relative light units intercepted by the luminometer in a one second interval.

2.4. Comparison of GlnLux Glutamine Accumulation between Crowded and Non-Crowded Maize Plants

At early growth stages, none of the indicators of in-season N health including *GlnLux* glutamine correlated with the N application rate (Table 1, Figure 1A). Young plants appeared to be N saturated even at the lowest N application rates. It was hypothesized that overplanting would deplete the applied N from soil to below a tissue-assimilate threshold that could be detected by *GlnLux*. Plot sections were overplanted in both 2015 and 2016 (Figure 3A). Crowding depleted *GlnLux* glutamine, significantly decreased the y-intercept (Figure 3B,C), and allowed differences in the N application rate to be resolved earlier, at V5 (Figure 3B,C), but not at V3 (data not shown). In general, crowding improved the correlation (in 2016, R^2 raised from 0 to 0.87), linearity and slope (away from zero) between *GlnLux* glutamine and N application rate at the earlier growth stage (Figure 3B,C).

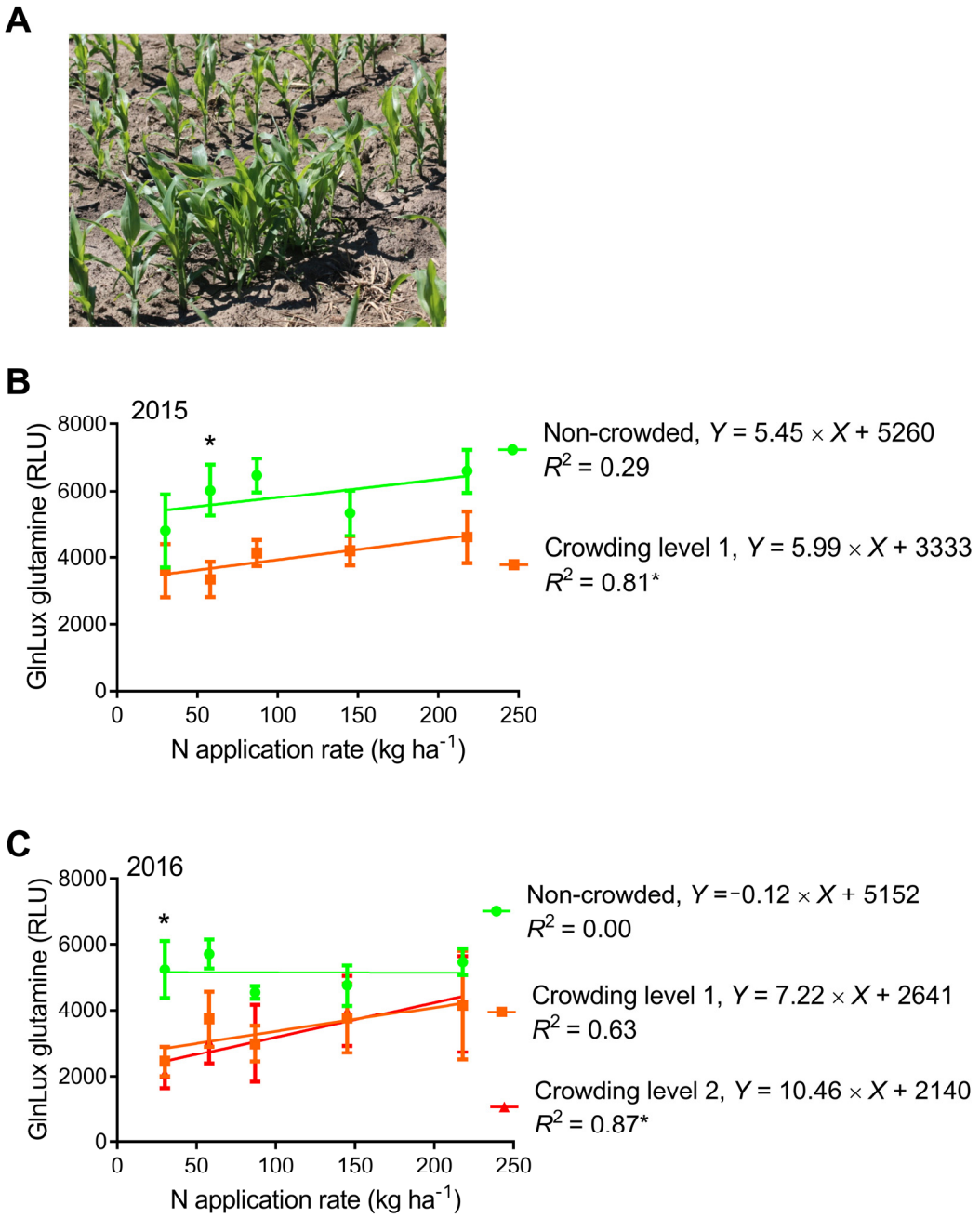

Figure 3. Increased maize planting density improves the correlation between early-season *GlnLux* glutamine output and N application rate. (**A**) Small sections (1 m in length) were overplanted within the main experimental plots; (**B**) An extra maize row was positioned along each side of the main row at planting, consisting of 20 extra plants in total (crowding level 1) in 2015; (**C**) In 2016 crowding level 1 was imposed, in addition to crowding level 2 consisting of 40 extra plants in total. (**B**,**C**) Plants from the main row were sampled for *GlnLux* glutamine content at the V5 growth stage, the results of which were plotted against N application rate. Each data point represents the mean of four replications (n = six pooled plants) ± SEM. Asterisks above the means represent significant differences between the non-crowded and crowded treatments with Šidák's multiple comparisons ($p < 0.05$). Asterisks beside R^2 values represent regressions with slopes that are significantly different ($p < 0.05$) from zero as determined with F tests. RLU, relative light units intercepted by the luminometer in a one second interval.

3. Discussion

Leaf Gln as a tool to assess available soil N and predict grain yield in a field environment has not previously been reported. Based on previous lab and greenhouse studies [54], it was hypothesized that leaf Gln at early-to–mid season, measured using the *GlnLux* biosensor, could report the N application rate and predict end-season grain yield in maize under field conditions.

3.1. GlnLux Glutamine Becomes a Reliable Indicator of N Application Rate as Vegetative Growth Progresses

The correlation between maize leaf *GlnLux* glutamine and the N application rate (Figure 1A–D) generally increased as vegetative growth progressed from V3 to V14, becoming most significant at or after V6. As maize and other cereal crop species mature, N demand increases along with the metabolic needs of the growing plant influenced by environmental factors [7,9,37,56]. The results presented here indicate that the modern maize hybrid tested has a developmental threshold for applied N, such that during early development (V3), N supply at the field site exceeded the demand for N-requiring macromolecules (e.g., protein) resulting in saturation of assimilatory free Gln, even at low N application (Table 1; Figure 1A). Another possibility is that at V3, the young plant roots may have not yet reached the mid-row where fertilizer was applied. However at or after V6, plants were able to uptake and assimilate N into Gln when higher soil N was available. Previous studies have shown that on average, ~40% of total seasonal N uptake occurs between the V6 and V14 growth stages [34,57]. The results of this study extend known temporal dynamics of N demand to include assimilatory Gln.

In the above description, Gln is simply a transitory molecule to deliver N which has been the focus of much previous research [43,58]. An alternative hypothesis is that maize plants maintain a pool of free N assimilate when local soil N supply exceeds the immediate demand of the plant. Such storage/accumulation of N has been termed "luxury consumption" [49,50]. In vegetative tissues, nitrate and protein are the N storage forms most reported, and of the latter, leaf ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the most abundant [18]. However, in oat and ryegrass, accumulation of free Gln and asparagine (another primary N assimilate) was observed in a situation where sulphur/copper deficiency slowed vegetative growth, causing N supply to exceed plant demand [49,50]. Accumulation of Gln was also observed in algae, and a rhizomatous alpine herb during excess N supply [51,59]. Moving forward, maize tissue concentrations of Gln, protein and nitrate need to be measured side by side in concert with growth rate metrics [18,60] in order to provide direct evidence of luxury Gln. For example, larger N rates should be applied to achieve a grain yield plateau, as did not occur in this report. A plateau with N left in the stover, or a yield decrease with more grain N concentration would show luxury N uptake during maximal yield. For now, the importance of luxury Gln remains unknown.

3.2. Measurements of GlnLux Glutamine at Different Growth Stages Correlate with End-Season Grain Yield

A key finding of this study is that free Gln in leaves, especially later in the growing season, is a good indicator of end-season grain yield traits in maize under N limiting conditions (Table 1; Figure 1E–H). Correlation of *GlnLux* glutamine and yield was also observed (Supplementary Materials Figure S3) in a distant relative of maize, notably finger millet (*Eleusine coracana* Gaertn.), a crop important in agroecosystems of the semi-arid tropics [61–63]. *GlnLux* glutamine content in finger millet leaves sampled 39 days after germination displayed tight correlation with grain yield across a range of N application rates (Supplementary Materials Figure S3C–E), conducted as part of a larger study in which N limitation effects on plant morphology were examined [64].

Grain yield traits may correlate to earlier stage free Gln in leaves primarily because that pool contributes to N-containing macromolecules (e.g., protein, chlorophyll, etc.), which can be remobilized during grain fill. During leaf senescence, chloroplasts are dismantled and channeled in a series of proteolytic reactions into several intermediates, eventually yielding Gln and other amino acids for remobilization to grain [43]. Maintaining a hypothetical pool of luxury Gln in leaves for future

remobilization into grain may bypass energetically-costly intermediates. Most vegetative N is stored in the stalk, followed by the leaves [34], and the proportional contribution of stalk N and leaf N to total vegetative N remobilized to the grain is roughly equal, at 45% each [65].

Leaf Gln is highest in youngest leaves (i.e., those at the top of the canopy; Supplementary Materials Figure S4; [54]) to enable growth, but in addition to its direct role in grain fill, it is interesting to speculate whether a leaf Gln pool may buffer against the senescence-associated scavenging of RuBisCO and other photosynthetic machinery leading to late-season declines in crop radiation use efficiency (RUE). N stress has been shown to decrease yield later in vegetative growth in part due to decreased RUE [66–68]. From a physiological perspective, maintaining a photosynthetically-active canopy late into the growing season is critical for maintaining high grain yields in maize [69]. Crops which maintain high levels of specific leaf nitrogen (SLN) over the growing season tend to have longer lasting canopies and higher RUE [70]. It has been hypothesized that higher SLN values in maize, while not increasing RUE directly, improve grain yield by acting as a buffer against post-silking N stress [71,72]. Furthermore, higher SLN values in maize improved NHI by reducing the flux of post-silking N uptake that goes to the leaves and increasing N flux to the grain [73]. It may be of interest to examine the free Gln pool in "stay-green" crop varieties, where the photosynthetic machinery is kept intact throughout much of grain-fill and senescence [74,75].

Whereas early-to-mid season leaf Gln correlated with yield, the correlation with end-season stover N percentage and content was variable (Table 1, Figure 1M–P). It may be that variable N remobilization during leaf senescence was responsible for this observation. An interplay of factors has been shown to contribute to variable remobilization of N from the stover during grain fill [65,73,76–78].

3.3. Comparison to Commercially Available In-Season N Health Indicators

Maize foliar chlorophyll, as determined with a SPAD meter, displayed a similar trend as *GlnLux* glutamine with respect to predicting grain yield (Figure 2A–F), although in 2014 the SPAD correlations were not significant at either the V6 or V12 growth stage (Figure 2D). This result suggests that foliar Gln content (determined with the *GlnLux* biosensor or otherwise) may be a more reliable predictor of yield, or at least equivalent, to SPAD chlorophyll. NDVI measurements (recorded with a GreenSeeker™ handheld instrument) only correlated with end-season grain yield in 2015 and 2016, and only at the V12 stage (Figure 2G–I). It has been suggested that reflectance-based technologies including GreenSeeker™ may be of limited use for early-season measurements of maize, because the instrument's field of view is large and includes soil as well as vegetation which is older, and less predictive of N status [79]. This was also shown here with Gln-yield correlation data (Supplementary Materials Figure S4). Maximum correlation using GreenSeeker™ is only achieved after adjustments are made according to the height of the plant canopy [80]. Additionally, the field-of-view is heavily dependent on the detector angle which can affect the correlation [81]. Here, in 2014 an additional experiment was performed to test the correlation between grain yield and FieldScout GreenIndex+™ (Spectrum Technologies Inc., Aurora, IL, USA) measurements, a smartphone application that uses leaf greenness to infer N health. The correlations were not significant at either V6 or V12 with respect to predicting grain yield (Supplementary Materials Figure S5). Therefore, *GlnLux* glutamine outperformed multiple commercially available leaf N-status tests in terms of predicting grain yield.

3.4. Can the Yield-Predictive Value of Leaf N-Health Indicators Be Improved by Creating Early-Crowding Subplots?

In a pilot experiment, early crowding significantly improved the early-stage correlation between *GlnLux* glutamine and the N application rate (Figure 3). A simple interpretation of this result is that a higher density of plants earlier in the season depleted the N applied to soil, preventing saturation of leaf N and hence free Gln. In future experiments, it would be interesting to explore whether such crowding can improve the efficacy of not only *GlnLux* glutamine, but also other N health indicators (e.g., SPAD chlorophyll) with respect to predicting grain yield. However, other plant crowding stresses

(e.g., diminished root growth per plant and quicker soil drying) may have also caused this response, indicating that foliar Gln may allow investigation of crop spacing effects, as well as other spatial interactions including weed stress.

3.5. Potential of Leaf Gln and GlnLux as Tools for Field Research

The data in this study show that free Gln in leaves is useful as a mid-season indicator of maize N status and for predicting grain yield with respect to soil N availability. Mid-season *GlnLux* measurements successfully predicted grain yield in a distantly related cereal (finger millet) as noted above, and in a preliminary experiment, in a different maize variety (Pioneer P9329, DuPont Pioneer, Johnston, IA, USA) grown in a different field (Supplementary Materials Table S1). However, it is important to note that this study examined Gln when N was the limiting factor; *GlnLux* glutamine may not be a good predictor of grain yield under other stress conditions. For example, glutamine synthetase has been shown in many plants to be affected by salt, drought, and pathogen stress [82]. However, in all three experimental years of this study, similar results were obtained despite significant weather stress including frost in 2015 (Supplementary Materials Figure S1B) and early-season drought in 2016 (Supplementary Materials Figure S1C). Nevertheless, future studies must be conducted at additional locations and experimental years to calibrate Gln results to different soil types, cropping histories, climates, and plant genotypes. Genotypic differences have been shown to exist in terms of N assimilatory dynamics, and there is presumably also variation in Gln accumulation [83–85]. Furthermore, care must be taken to sample plants for Gln at a similar time of day, to overcome diurnal regulation of glutamine synthetase and other enzymes of N metabolism [86–88].

Although *GlnLux* glutamine was demonstrated in this study to reflect N status at various points in the growing season, integration with other measures/models [34–42] is required to generate an estimation of N requirement, and prescribe a mid-season application rate. The results of this study displayed consistent correlation of *GlnLux* glutamine with end-season yield, but in the future it may be interesting to test the leaf lamina instead of the midrib. As the midrib can be variable in size, this may result in variability in the mass of tissues sampled. Tissue mass could also be standardized by weight. In this report, care was taken to sample a consistent position of the leaf and to pool multiple samples to reduce variability. Additionally, the *GlnLux* test is currently lab-based and requires shipping of frozen leaf punches. A simpler method of Gln detection (improved protocol or alternative technology), ideally on-farm, would facilitate its utilization by growers and researchers. Though *GlnLux* glutamine better predicted grain yield compared to GreenSeeker^{TM} NDVI, the latter technology is easier to use.

Apart from its yield predictive value, *GlnLux* glutamine may also be a useful tool for understanding basic plant physiology at the field level. The pool of free assimilatory Gln might be more responsive than chlorophyll (e.g., SPAD) to sudden changes in soil N availability and might reveal conditions that spike or suppress Gln rapidly. The effect of precipitation and temperature on nitrate leaching or denitrification, and resulting free Gln levels could be examined with daily sampling throughout the season. *GlnLux* could allow examination of the N physiology of individual plants under field conditions at extremely fine temporal resolution, as well as spatial resolution [54].

4. Materials and Methods

4.1. Main Experiment: Site Description and Planting

In 2014, 2015, and 2016, treatments were imposed onto and data collected from a long-term nitrogen (N) field experiment that was initiated in 2008 at the University of Guelph Elora Research Station (lat. 43°39′ N, long. 80°25′ W, 376 m elevation), Ontario, Canada. The field is described as a Guelph loam (fine loamy, mixed, mesic Glossoboric Hapludalf, or orthic grey brown luvisol) [89], pH 7.7, silt 48%, clay 20%, soil organic matter 4.5% (analyzed by Agri-Food Laboratories Inc., Guelph, ON, Canada). The 30-year mean annual precipitation is 871 mm, and average annual air

temperature is 6.7 °C [55]. Additional daily climatic data recorded during the experimental period is displayed in Supplementary Materials Figure S1. Moldboard plowing was performed in the fall of each year, and spring cultivation before planting. Maize (*Zea mays* L., variety Dekalb DKC39-97RIB, glyphosate tolerant, 2700 Ontario Corn Heat Units; Monsanto Canada Inc., Winnipeg, MB, Canada) was machine planted (79,000 seeds ha^{-1}) in a randomized complete block design (RCBD) with four replications. The trial was divided into experimental units consisting of six-row plots with 0.76 m row spacing and 17 m row lengths. Only the four centre rows of each plot were used for sampling and measurements. Planting was performed on 9 May (2014), 6 May (2015), and 6 May (2016).

4.2. Crowding Experiment: Planting

In 2015 and 2016, 1 m long sections were overplanted within each main plot. An additional maize row 1 m in length of the same variety was positioned 5 cm along each side of the main row, consisting of 10 plants per extra row (crowding level 1, CL1) in 2015. In 2016, crowding level 1 was imposed in addition to crowding level 2 (CL2), consisting of 20 plants per extra row. In each plot and year, two sections of each crowding level were overplanted. These extra plants were removed from the field at stage V5 (five leaves fully emerged).

4.3. Fertilizer and Herbicide Treatments

Urea was provided as starter N fertilizer to all plots (30 kg N ha^{-1}) through the planter, 5 cm beside and below seeding depth. Additionally, five different fertilizer N treatments of 0, 28, 57, 115, and 188 kg ha^{-1} were injected via an applicator fitted with 1.27 cm wavy coulters (Demco, 558 L, Kent Farm Supply, Blenheim, ON, Canada) mid-row to a 5–10 cm depth as urea-ammonium-nitrate (UAN) solution shortly after planting. Including the starter, total N application rates were therefore 30, 58, 87, 145, and 218 kg ha^{-1}. In each replication year sampled, the previous year was maize grown under an N rate which was constant across all treatment plots. Five soil cores were sampled from the top 30 cm of each plot before N application and sent for analysis (Agri-Food Laboratories Inc.) of KCl extractable NH_4/NO_3-N. Potassium and phosphorus were applied at non-limiting rates as needed with band application at seeding or before tillage with broadcast application (0-46-0 and 0-0-60). Post-emergence weed control was performed by spraying mesotrione (0.3 L ha^{-1}; Callisto, Syngenta, Guelph, ON, Canada), and S-metolachlor/atrazine (3.5 L ha^{-1}; Primextra-II-Magnum, Syngenta) on 19 May (2014) and 14 May (2015). No pre-emergent herbicide was applied in 2016. Glyphosate was applied as needed (Roundup WeatherMAX or Transorb, Monsanto, Creve Coeur, MO, USA).

4.4. End-Season Measurements

Plot harvest occurred on 30 October (2014), 3 November (2015), and 25 October (2016). Mechanical harvesting was performed with a four-row GleanerR42 rotary combine (AGCO Corporation, Duluth, GA, USA) and monitored with a GrainGage (HarvestMaster, Juniper Systems, Inc., Logan, UT, USA) for assessing grain weight. Moisture was adjusted to 15.5% for analysis of grain yield. Five representative plants per plot (manually harvested, and dried in an oven at 80 °C until constant weight) were used to obtain biomass and total N percentage with Dumas combustion [90] of above-ground plant tissue. Ear dry weight consisted of the grain, cob, and husk. Stover consisted of the cob and husk along with the shoot tissue. Harvest index (HI) was determined by dividing grain dry weight by the grain plus stover dry weight. Nitrogen harvest index (NHI) was determined by dividing total grain N content by the total grain plus stover N content.

4.5. Relative Measurements of Glutamine from Leaf Disk Extracts

Leaf tissue disks (6.35 mm in diameter) were sampled with a hole-punch tool (Fiskars Brands Inc., Middleton, WI, USA) along the midrib at several growth stages (Supplementary Materials Figure S2A–D): V3 (three leaves including the leaf collar have fully emerged from the plant); V6 (six leaves fully emerged); V12 (twelve leaves fully emerged); V14 (fourteen leaves fully emerged).

At each stage, six subsample leaf disks from separate plants in each plot were harvested, immediately frozen in liquid N_2, and stored at −80 °C until processing. Sampling of the overplanted plot sections was performed at V3 and V5 (five leaves fully emerged) for comparison to non-crowded plants. Sampling of all growth stages was performed at the same time of day to control for any effects of circadian rhythm.

Leaf disks were analyzed for glutamine (Gln) content with the *GlnLux* biosensor (Supplementary Materials Figure S2E) [53]. The six leaf disk subsamples from each plot were pooled into 200 µL of 0.1% chilled protease inhibition cocktail (PIC) (P9599-1ml, Sigma-Aldrich, St. Louis, MO, USA) and homogenized with an automated tissue disruptor (Bead Ruptor 12, Omni International, Kennesaw, GA, USA). The homogenate was then diluted six-fold in 0.1% chilled PIC and centrifuged (model 5415R, Eppendorf, Hauppauge, NY, USA; 4 °C, 20 min, 13,000 rpm). The resulting plant tissue extract supernatant was further diluted ten-fold in 0.1% chilled PIC and stored at −20 °C until analysis. Low temperatures and PIC were utilized throughout the protocol to ensure accurate detection of the free portion of plant Gln (i.e., not liberated from protein/peptide breakdown which may otherwise occur during the protocol). Analysis of the extract was conducted with the *GlnLux* whole-cell biosensor as previously described [53,54]. Briefly, *GlnLux E. coli* cells were co-incubated with the plant tissue extracts, and then luminescence outputs from 96-well plates were measured using a luminometer (MicroLumatPlus, Berthold Technologies, Bad Wildbad, Germany) resulting in relative measurements of plant tissue Gln ("*GlnLux* glutamine").

To optimize the protocol for use in the field, different maize leaves (old leaves versus younger, more recently emerged leaves), and different positions along the midrib of the younger leaf were tested for correlation between *GlnLux* glutamine and N application rate (Supplementary Materials Figures S4 and S6). *GlnLux* glutamine from young, recently emerged leaves sampled at a position near the base strongly correlated with the N application rate. In a greenhouse experiment, it was previously determined that *GlnLux* glutamine from young leaf tissue best correlates with the N fertilization status of maize seedlings [54]. Therefore, young tissue was used for all subsequent analysis. Specifically, at all growth stages in this study, the leaf sampled for *GlnLux* glutamine was second from the top of the plant, at a consistent position near the base of the leaf. Additional optimization included ensuring that *GlnLux* output was not significantly affected by sampling different young leaves (Supplementary Materials Figure S7), as plants receiving the extremes of the N application range initiated leaves at slightly different rates.

4.6. SPAD and GreenSeeker™ Measurements

Measurements were recorded at the V6 and V12 growth stage. Maize foliar chlorophyll concentration was estimated with a Soil Plant Analysis Development (SPAD) meter (SPAD-502, Konika Minolta, Tokyo, Japan). SPAD measurements were recorded at five positions from the uppermost maize leaf of five (2014) or three (2015 and 2016) representative plants per plot, and then averaged for each replicate. Normalized Difference Vegetation Index (NDVI) was recorded with a GreenSeeker™ handheld crop sensor (Trimble, Westminster, CO, USA) within 2 h of noon by scanning and averaging over the canopy of two (2014) or three (2015 and 2016) separate 4 m long row segments per plot.

4.7. Statistical Analysis

Data outliers were identified and removed with ROUT, Q = 1% [91]. Pearson correlation was used to compare normalized vegetative growth stage *GlnLux* glutamine levels (raw luminometer output—the luminometer output of a negative control consisting of 0.1% PIC in the place of tissue extract) to N application rate and end-season field measurements. Pearson correlation of SPAD chlorophyll and GreenSeeker™ NDVI with yield was also performed. *GlnLux* glutamine data from crowded and non-crowded sections was regressed linearly against the N application rate. F tests were used to examine if slopes deviated from zero, and if the intercepts of the equations differed

significantly [92]. Significant differences between *GlnLux* glutamine levels from the crowded and non-crowded treatments within individual N application rates were tested with Šidák's multiple comparisons [93]. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA), and significance was determined at $p < 0.05$, 0.01, 0.001, or 0.0001 as indicated.

5. Conclusions

The results of this study show that at early-to-mid season, free Gln in leaves as measured using the *GlnLux* biosensor could report the prior N application rate and predict end-season grain yield in maize under field conditions. Free Gln in leaves may serve to explore basic questions of plant physiology at the field level, including whether free leaf Gln may improve yields by acting as an N reservoir during leaf N remobilization, and buffer against declines in radiation use efficiency during grain fill. If integrated with other measures and models, leaf Gln may hold potential as a tool for site-specific N management by reporting soil N availability and predicting grain yield.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/7/2/41/s1, Figure S1: Daily precipitation (mm), high, and low temperatures (°C) during the growing season at the experimental site in 2014, 2015 and 2016. Figure S2: Methodology of field sampling and the *GlnLux* assay, Figure S3: Correlation of early-season *GlnLux* glutamine and end-season grain yield in finger millet (*Eleusine coracana* Gaertn.), Figure S4: Maize leaf tissues sampled from young leaves within the same vegetative growth stage display increased *GlnLux* glutamine correlation with grain yield as compared to older leaves, Figure S5: Pearson correlation of end-season maize grain yield (kg ha^{-1}) and FieldScout GreenIndex+^{TM} (Smartphone application) output, Table S1: Pearson correlation between maize *GlnLux* glutamine (alternate variety Pioneer P9329) and end-season measurements at an alternate field location in 2014, Figure S6: Maize leaf tissue sampled from different positions along the midrib of a young leaf (located second from the top of the plant, not fully emerged) displays increased *GlnLux* correlation with grain yield towards the base of the leaf compared to more distal locations, Figure S7: *GlnLux* glutamine correlation with grain yield shows a slight, but insignificant, increase when standardized by leaf number at silking (R1) since individual plants grew at slightly different rates.

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Conflicts of Interest: A US Patent has been issued on the *GlnLux* biosensor technology (US 61/499286) and conflict of interest can be present, but the technology is not currently commercialized.

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