A Biosensor-Based Leaf Punch Assay for Glutamine Correlates to Symbiotic Nitrogen Fixation Measurements in Legumes to Permit Rapid Screening of Rhizobia Inoculants under Controlled Conditions

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Legumes are protein sources for billions of humans and livestock. These traits are enabled by symbiotic nitrogen fixation (SNF), whereby root nodule-inhabiting rhizobia bacteria convert atmospheric nitrogen (N) into usable N. Unfortunately, SNF rates in legume crops suffer from undiagnosed incompatible/suboptimal interactions between crop varieties and rhizobia strains. There are opportunities to test much larger numbers of rhizobia strains if cost/labor-effective diagnostic tests become available which may especially benefit researchers in developing countries. Inside root nodules, fixed N from rhizobia is assimilated into amino acids including glutamine (Gln) for export to shoots as the major fraction (amide-exporting legumes) or as the minor fraction (ureide-exporting legumes). Here, we have developed a new leaf punch based technique to screen rhizobia inoculants for SNF activity following inoculation of both amide exporting and ureide exporting legumes. The assay is based on measuring Gln output using the GlnLux biosensor, which consists of Escherichia coli cells auxotrophic for Gln and expressing a constitutive lux operon. Subsistence farmer varieties of an amide exporter (lentil) and two ureide exporters (cowpea and soybean) were inoculated with different strains of rhizobia under controlled conditions, then extracts of single leaf punches were incubated with GlnLux cells, and light-output was measured using a 96-well luminometer. In the absence of external N and under controlled conditions, the results from the leaf punch assay correlated with $^{15}$N-based measurements, shoot N percentage, and shoot total fixed N in all three crops. The technology is rapid, inexpensive, high-throughput, requires minimum technical expertise and very little tissue, and hence is relatively non-destructive. We compared and contrasted the benefits and limitations of this novel diagnostic assay to methods.

Keywords: legume, symbiotic nitrogen fixation, nodule, rhizobia, biosensor, glutamine
INTRODUCTION

Legumes are critical to human and livestock agricultural systems (Broughton et al., 2003; Leigh, 2004; Foyer et al., 2016), as they fix atmospheric nitrogen (N) into a usable form of N (NH$_3$/NH$_4^+$) through symbiotic association with rhizobia bacteria inside underground root nodule organs (Udvardi and Poole, 2013). The enzyme glutamine (Gln) synthetase assimilates NH$_4^+$ into amino acids (Bernard and Habash, 2009; Betti et al., 2012). Therefore, symbiotic nitrogen fixation (SNF) provides the essential building block for amino acid biosynthesis (Prell and Poole, 2006; Udvardi and Poole, 2013), enabling legumes such as lentil and soybean to be a primary source of high-quality protein for billions of people especially in developing nations, and for livestock especially in wealthier societies (Graham and Vance, 2003).

SNF also enables the deposition of organic fertilizer into soil during litter decomposition (Thilakarathna et al., 2015, 2016a), thus reducing the need for synthetic N fertilizers, of which subsistence farmers are primary beneficiaries. Unfortunately, some legume species and varieties show low SNF rates, in part due to incompatible or suboptimal interactions with strains of available soil rhizobia (Thilakarathna and Raizada, 2017). Although improved rhizobia can be introduced, screening a panel of rhizobia strains against local crop varieties/landraces for improved SNF is expensive especially for subsistence farmers in developing nations, in part because of the limitations of current SNF diagnostic methods (Unkovich and Pate, 2000; Howieson and Dilworth, 2016). Symbiotic nitrogen fixation activity is currently measured using different methods including counting the number of differentiated rhizobia (bacteroids) per nodule (Bourcy et al., 2013), N difference assay (Unkovich et al., 2008), ureide assay (Unkovich et al., 2008), acetylene reduction assay (Lodwig et al., 2003; Starker et al., 2006), hydrogen production (Kiers et al., 2003; Cabeza et al., 2015), and $^{15}$N techniques (Lodwig et al., 2003), of which $^{15}$N is the most commonly used and considered to be the most accurate (Howieson and Dilworth, 2016). These methods are challenging in terms of time, labor, accuracy, and the need for a non-fixer reference plant, destructive sampling, large amounts of tissue, technical expertise, expensive reagents, and/or equipment (Herridge et al., 2008; Howieson and Dilworth, 2016). Therefore, there is need for alternative high-throughput SNF diagnostic methods.

Inside root nodules, fixed N from rhizobia is assimilated into Gln (Prell and Poole, 2006; Tegeder, 2014; Figure 1A) for export to shoots as amino acids as the major fraction (amide-exporting legumes) or as the minor fraction (10–20% in ureide-exporting legumes) (Pate et al., 1980; Atkins, 1987). Generally, legumes that originate in temperate regions (e.g., alfalfa, pea, clover) are amide exporters, whereas those that originate in the tropics and subtropics (e.g., soybean, cowpea) predominate export fixed N as ureide compounds (e.g., allantoin) (Unkovich et al., 2008). Whole cell biosensors have been engineered to detect metabolites and emit outputs in response (e.g., fluorescence, luminescence) that can be easily measured, substituting for more expensive analytical chemistry methods such as liquid chromatography mass spectrometry (LC-MS) (Goron and Raizada, 2014). We previously developed a rapid leaf punch-based assay for Gln using a whole cell Gln biosensor called GlnLux (Tessaro et al., 2012). GlnLux was created by transforming an Escherichia coli Gln auxotroph with a constitutive lux reporter (Tessaro et al., 2012). Advantages of an auxotroph compared to expression-based reporter fusions are that there is an absolute requirement for the metabolite (i.e., Gln) and significantly fewer artifacts caused by microbial or plant metabolism or signaling. We showed that GlnLux cells exposed to extracts of leaf punches taken across the veins of growing leaves can accurately report N availability in maize (Tessaro et al., 2012; Goron and Raizada, 2016; Goron et al., 2017).

Here, we tested the hypothesis that the GlnLux leaf punch assay can be used to infer SNF output for high-throughput screening of legume inoculants under controlled conditions with minimal exogenous N (Figure 1A). Extracts of single leaf punches spanning veins of young legume leaves were incubated with GlnLux cells in 96-well liquid culture plates, then photon emissions were measured using a plate luminometer (Figure 1B). In order to validate the GlnLux leaf punch bioassay, two approaches were used: evaluation of different rhizobia strains using both amide and ureide exporting legumes. The crops used were subsistence Nepalese varieties of lentil (Lens culinaris) (an amide exporter), a legume of critical importance to South Asia, the Middle East, and East Africa (Sharpe et al., 2013); cowpea (Vigna unguiculata L. walp.) (a ureide exporter), a drought-tolerant legume grown in Sub-Saharan Africa, South Asia, and the Caribbean (Ehlers and Hall, 1997); and soybean (Glycine max) (a ureide exporter), the world’s most important legume in terms of production (Herridge et al., 2008). The results of GlnLux were compared to traditional SNF diagnostic methods including the standard $^{15}$N dilution technique. Plants were grown with minimal N fertilizer to facilitate N-isotope analysis.

MATERIALS AND METHODS

Plant Materials and Rhizobium Strains

For the lentil × rhizobia strain experiment, the crop variety was Nepalese Simal, and there were four Rhizobium leguminosarum biovar viciae strains (wild type: 3841, VF39, 248; mutant 17-B) (Yost et al., 2006; Vanderlinde et al., 2011). For the cowpea × rhizobia strain experiment, the crop variety was Nepalese Surya and there were two strains of Bradyrhizobium yuanmingense (TTC9, TSC10), one strain of B. japonicum (DTB4) and one strain of B. elkanii (DTC9) (Sarr et al., 2009). For the soybean × rhizobia strain experiment, the crop variety was Nepalese Surya and there were two strains of Bradyrhizobium yuanmingense (TTC9, TSC10), one strain of B. japonicum (DTB4) and one strain of B. elkanii (DTC9) (Sarr et al., 2009). For the soybean × rhizobia strain experiment, the crop variety was Nepalese Surya and there were two strains of B. japonicum (USDA 110, USDA 123, USDA 510) (Sadowsky et al., 1987; Bhagwat et al., 1991; Kaneko et al., 2002) and two strains of B. elkanii (USDA 94, USDA 76) (Kuykendall et al., 1992). Different rhizobia strains for each crop were selected to capture a diversity of nodulation and N fixation efficiency.

Plant Growth Conditions and Treatments

Seeds of all three crops were surface-sterilized with 70% ethanol for 2 min, 4% sodium hypochlorite for 3 min, and washed...
within a plastic container. There were eight trays (in individual germination pouches) were randomly allocated to the study, five rhizobia treatments and the non-inoculated control. Different rhizobia treatments were not placed within the same container to avoid cross contamination of rhizobia strains. There were three containers per treatment, which were continuously randomized within and across light shelves. For the soybean species had a negative control, comprised of plants that were not inoculated with rhizobia.

**GlnLux Leaf Punch Sampling**

Eight healthy representative plants per treatment were sampled from lentil, cowpea, and soybean 4 weeks after inoculation with rhizobia as indicated. Leaf punches were collected from the fully expanded youngest leaf, across the leaflet main vein from lentil (from one leaflet at the top), cowpea, and soybean using a 3 mm (lentil) and 6.35 mm (cowpea and soybean) hand puncher (235270975; Fiskars Brands Inc., Middleton, WI, United States) and hand puncher (235270975; Fiskars Brands Inc., Middleton, WI, United States) and
immediately frozen in liquid N. Leaf punches were stored at −80°C.

Plant Morphometric Analysis
The same eight plants per treatment harvested for GlnLux measurements were used. The number of nodules per plant was counted manually. Roots were scanned using an Epson Expression 1640 scanner (Epson Canada Ltd., Markham, ON, Canada), and a detailed root morphological analysis was undertaken using WinRHIZO software (Regent Instruments Inc., Quebec City, QC, Canada) including root volume, total length, surface area, and average diameter. Shoot and root dry weights were measured after drying the plant materials in an oven at 60°C for 3 days.

Shoot Nitrogen and $^{15}$N Analysis
The same eight plants per treatment harvested for GlnLux measurements were used. The dried shoot samples (see above) were ground using a coffee grinder followed by a Bead Ruptor 12 Homogenizer (OMNI International, Kennesaw, GA, United States) and analyzed for $^{15}$N and total N% using a mass spectrometer (Costech ECS4010 Elemental Analyzer coupled to a Delta V mass spectrometer, Costech, CA, United States) at the Stable Isotope Facility, University of Saskatchewan, and University of British Columbia, Canada, using a standard protocol (Thilakarathna et al., 2012, 2016b). The %Ndfa of the lentil and soybean was calculated using the following formula according to the isotope dilution technique:

$$\% \text{Ndfa} = \left(1 - \frac{\text{atom } % \text{N excess}_{\text{lentil/soybean-inoculated}}}{\text{atom } % \text{N excess}_{\text{lentil/soybean-uninoculated}}} \right) \times 100,$$

where atom % $^{15}$N excess = atom % $^{15}$N$_{\text{lentil/soybean}}$ − 0.3663.

The amount of shoot N derived from SNF was calculated based on the shoot N content and %Ndfa (shoot N content × %Ndfa/100).

Biosensor Strain
Construction of the GlnLux biosensor strain was previously reported (Tessaro et al., 2012). Briefly, a Gln- auxotrophic E. coli strain (JW3841-1, KanR) was generated by inserting a kanamycin cassette into GlnA [glnA732(del)::kan] (Baba et al., 2006). The strain was subsequently transformed with ampicillin-resistant plasmid pT7-lux (Meighen and Szittner, 1992) which contained a constitutive T7 promoter from Xenorhabdus luminescens driving expression of the luxCDABE operon from Vibrio fischeri to create strain GlnLux.

GlnLux Bacterial Growth Media
GlnLux bacteria were cultured in LB medium, which consisted of 5 g/l NaCl (BP358-212, Fisher Scientific), 5 g/l yeast extract (DF0127179, Fisher Scientific), and 10 g/l tryptone (BP1421-500, Fisher Scientific), with or without 12 g/l Bacto-Agar (BD; DF0140010, Fisher Scientific), pH 7.2. M9 minimal medium consisted of 20 ml/l 20% (w/v) D-(+)-glucose (G5767, Sigma), 100 µl/l 1 M CaCl$_2$ (C-79, Fisher Scientific), 2 ml/l 1 M MgSO$_4$ (230391, Sigma), and 200 ml/l 5 × M9 salts (A-0171, Sigma), pH 7.0. All liquid and solid plate media were supplemented with 50 µg/ml kanamycin monosulfate (K378, PhytoTech, United States) and 100 µg/ml carbenicillin disodium salt (C346, PhytoTech, United States) to select for the disrupted glnA chromosome and reporter plasmid, respectively.

Leaf Punch Luminometer Assays – GlnLux Cell Preparation
Cells were prepared as previously described (Tessaro et al., 2012). Briefly, GlnLux bacteria were inoculated into 15 ml of LB medium in a 50 ml Falcon tube, and incubated overnight at 37°C with shaking at 250 × g. The culture was spun down at 2500 × g at 21°C for 10 min and the supernatant was decanted. The culture was then washed 3 × in sterile M9 minimal medium with centrifugation as above. Finally, the culture was resuspended in 15 ml sterile M9 minimal medium in a 50 ml Falcon tube, and the GlnLux density was adjusted to OD$_{595}$ = 0.025 using sterile M9 minimal medium. The resuspended GlnLux culture was incubated at 37°C with shaking at 250 × g for 14 h to deplete any endogenous Gln.

GlnLux Leaf Punch Luminometer Measurements of Plant Gln
The procedure was adapted from a previous protocol (Tessaro et al., 2012). Individual frozen leaf punches were ground in a 2 ml conical bottom microcentrifuge tube placed on ice using a micropestle (K749515000, Kimble Chase, Fisher Scientific) with two ml/l 0.1% final (v/v) protease inhibitor cocktail (PIC) for plant cell extracts (100% stock; #P9599, Sigma). Plant extracts were centrifuged for 20 min at 4°C at 13,000 × g, and the supernatant was transferred to a microcentrifuge tube placed on ice. The plant extracts were diluted 100-fold in ddH$_2$O. The diluted plant extracts were used for luminometer assays.

For luminometer assays, white opaque 96-well reader plates (807-200-589, Fisher Scientific) were loaded with 160 µl/well M9 minimal medium followed by 20 µl/l of each plant extract. Finally, 20 µl of 14 h-Gln-depleted GlnLux culture (pre-depletion OD$_{595}$ = 0.025) was added to each well. Plates were sealed with non-breathable sterile film (#361006008, Fisher Scientific) were loaded with 160 µl/well M9 minimal medium followed by 20 µl/l of each plant extract. Finally, 20 µl of 14 h-Gln-depleted GlnLux culture (pre-depletion OD$_{595}$ = 0.025) was added to each well. Plates were sealed with non-breathable sterile film (#361006008, Fisher Scientific) to prevent media evaporation, and centrifuged for 5 s at 2000 × g to mix the GlnLux bacteria with plant extracts and M9 minimal medium. The plates were incubated at 37°C without shaking. For lux quantification, plates were read in a MicoLumatPlus LB96V luminometer with WinGlow Software (Berthold Technologies, Germany). Samples were read after 2 h of incubation for 1 s in a luminometer chamber temperature of 37°C in an endpoint assay using the integrate software function. Readings were taken every hour after incubation until lux values saturated. The plates were transferred back and forth from a 37°C incubator, and non-breathable films were replaced following each read. The 0 µg/ml Gln standard reading was subtracted from all lux values.

To test the biosensor response to exogenous Gln, six different concentrations of Gln standards (1-Gln G229, PhytoTech, 0,
RESULTS

Leaf Punch Screening of Inoculants Applied to Lentil

To test the GlnLux leaf punch assay with lentil, four strains of *R. leguminosarum* bv. *viciae* (VF39, 248, 3841, and 17-B), which differ in their N-fixation capacities, were inoculated onto a variety of lentil grown by smallholder Nepalese hillside farmers (*Figure 2A*). The different rhizobia strains all produced a similar number of root nodules (*Figure 2B* and Supplementary Figure S2A). A single punch per plant from a young leaf (from low-N grown lentils) was ground in PIC, co-incubated for 2 h with GlnLux cells in 96-well plates, and then the lux signal quantified after a 2-min read (*Figure 1B*). The GlnLux output (*Figure 2E*) showed a similar trend as the shoot N% (*Figure 2F*), and the percentage N derived from the atmosphere (%Ndfa) (*Figure 2G*). GlnLux output strongly correlated with SNF measured using the $^{15}$N stable isotope assay (%Ndfa) (Pearson correlation coefficient $R^2 = 0.9972$) (*Figure 2H*), plant shoot N% ($R^2 = 0.8910$) (*Figure 2I*), and amount of shoot N fixed ($R^2 = 0.890$) (*Figure 2J*). The GlnLux result was not an artifact of altered Gln demand, as significant differences were not observed among the different rhizobia treatments for normalized shoot dry weight (*Figure 2C*), normalized root dry weight (*Figure 2D*), normalized total dry weight, shoot/root biomass ratio, root length, root surface area, or volume (Supplementary Figures S2B–F).

Leaf Punch Screening of Inoculants Applied to Cowpea

To test the GlnLux leaf punch assay with cowpea, a ureide exporter, two strains of *B. yuanmingense* (TTC9 and TSC10), one strain of *B. japonicum* (DTB4) and one strain of *B. elkanii* (DTC9), which differ in their nodulation capacity, were
inoculated onto a variety of cowpea grown by smallholder Nepalese hillside farmers (Figure 3A). The different rhizobia strains produced a significantly different number of root nodules ($P < 0.0001$) (Figure 3B and Supplementary Figure S3A). The GlnLux output showed a similar trend (Figure 3E) as the shoot N% (Figure 3F) and the %Ndfa (Figure 3G). GlnLux output correlated with SNF measured using the $^{15}$N stable isotope assay (%Ndfa) (Pearson correlation coefficient $R^2 = 0.7038$) (Figure 3H), plant shoot N% ($R^2 = 0.9873$) (Figure 3I), and amount of shoot N fixed ($R^2 = 0.8641$) (Figure 3J). Significant differences were not observed among the different rhizobia treatments for normalized root dry weight (Figure 3D), root length, root surface area, or volume (Supplementary Figures S3B–D). Plants inoculated with strain TTC9 had the highest shoot (Figure 3C) and total biomass (Supplementary Figure S3E), whereas the lowest biomass and shoot/root ratio were associated with strain DTC9 (Supplementary Figures S3E,F).

Leaf Punch Screening of Inoculants Applied to Soybean
To test the GlnLux leaf punch assay with soybean, another ureide exporter, a Nepalese subsistence farmer variety of soybean was inoculated with three strains of $B$. $yuanmingense$ (TTC9 and TSC10), one strain of $B$. $japonicum$ (DTB4), one strain of $B$. $ekanii$ (DTC9), and the non-inoculated control. (B) Number of nodules per plant. The non-inoculated control had no nodules. (C) Normalized shoot dry matter per plant. Normalization of plant dry matter (rhizobia inoculated) was achieved by dividing dry matter of the non-inoculated plants. (D) Normalized root dry matter per plant. (E–J) Validation of the GlnLux lumino meter assay as a method to measure SNF in a ureide exporting legume (cowpea) by comparing results to total N% and $^{15}$N dilution methods. (E) Corresponding GlnLux leaf punch outputs. A 0 µg/ml Gln standard reading was subtracted from all lux values, which were read in randomized replicates. RLU, relative lu units. (F) Shoot total N% analyzed using a mass spectrometer. (G) Percentage of N derived from the atmosphere (%Ndfa) calculated using the isotope dilution technique (see section Materials and Methods). (H) Correlation between GlnLux and %Ndfa (Pearson $R^2$). (I) Correlation between GlnLux and shoot total N%. (J) Correlation between GlnLux and amount of shoot N fixed. For all graphs, the error bars represent the SEM ($N = 8$). The different letters on top of each histogram indicate significant differences in the mean between treatments.

**DISCUSSION**

During the 1980s, the close relationship between xylem ureide composition and SNF activity was used to develop the well-known ureide assay, a technique to measure SNF in ureide exporting legumes (Herridge, 1982; Herridge and Peoples, 1990). $^{15}$N-methods have been used to validate the ureide method for SNF measurement (Herridge and Peoples, 1990). Similarly, here we have developed a new leaf punch-based technique to
FIGURE 4 | The GlnLux leaf punch assay to measure SNF in soybean. (A–D) Effect of rhizobia inoculants on morphological traits. (A) Representative pictures of soybean plants inoculated with three strains of B. japonicum (USDA 110, USDA 123, 510) and two strains of B. elkanii (USDA 94, USDA 76) and the non-inoculated control. (B) Number of nodules per plant. (C) Normalized shoot dry matter per plant. Normalization of plant dry matter (rhizobia inoculated) was achieved by dividing dry matter of the non-inoculated plants. (D) Normalized root dry matter per plant. (E–J) Validation of the GlnLux luminometer assay as a method to measure SNF in a ureide exporting legume (soybean) by comparing results to total N% and 15N dilution methods. (E) Corresponding GlnLux leaf punch outputs. A 0 µg/ml Gln standard reading was subtracted from all lux values, which were read in randomized replicates. RLU, relative lux units. (F) Shoot total N% analyzed using a mass spectrometer. (G) Percentage of N derived from the atmosphere (%Ndfa) calculated using the isotope dilution technique (see section Materials and Methods). (H) Correlation between GlnLux and %Ndfa (Pearson R²). (I) Correlation between GlnLux and shoot total N%. (J) Correlation between GlnLux and amount of shoot N fixed. For all graphs, the error bars represent the SEM (N = 8). The different letters on top of each histogram indicate significant differences in the mean between treatments.

screen rhizobia inoculants for SNF activity following inoculation of both amide exporting (lentil) and ureide exporting (cowpea, soybean) legumes. The assay is based on measuring Gln output using the GlnLux biosensor, with minimal external N under controlled conditions. SNF-output as inferred from the GlnLux assay strongly correlated with the proportion of N derived from SNF measured using the 15N dilution method in both lentil (Figures 2H,J) and soybean (Figures 4H,J); the correlation for cowpea was lower but still strong (R² = 0.7038) (Figures 3H,J). The cowpea result may have been due to the four selected rhizobia strains which represented extremes in SNF activity, either high (TSC-10 and TTC-9) or low (DTB-4 and DTC-9), with no intermediate strain(s) (Figure 3B). The SNF capacity of a legume can vary due to the node number and/or N fixation activity of a particular rhizobia strain – host genotype combination (Thilakarathna and Raizada, 2017). In lentil, differences in SNF output by the rhizobia were caused by differential SNF activity rather than nodulation (Figures 2B,G). In cowpea, SNF likely varied due to the dissimilar number of nodules observed between inoculants (Figures 3B,G), but we cannot rule out differences in SNF activity. In soybean, differences in SNF output were caused by differences in rhizobia activity and/or nodulation ability, as some inoculants (e.g., USDA 94) had high nodulation but apparent low fixation (Figures 4B,G).

We have compared the efficacy of the GlnLux method against the currently available methods to quantify SNF (Table 1). The GlnLux leaf punch test could be performed on juvenile plants, and bypassed the need for 15N or tissue-N analyses under the conditions used. As the biosensor and resulting assay are highly sensitive (<1 nM Gln), measurements of Gln concentrations require only leaf punches (Tessaro et al., 2012; Goron and Raizada, 2016), and in fact, in this study, single leaf punch extracts were diluted 1/100th prior to the assay. The leaf punch test is thus relatively non-destructive, and furthermore the space requirement to store samples is minimal. Hundreds of leaf punches can be assayed in a single day by one individual with minimal training, especially with automated bead-based grinding of leaf tissue. In our experience, leaf punches can be stored in −80°C for more than 1 year for later GlnLux analysis. All reagent costs are minimal, resulting in an assay cost of only ~$1 USD per sample, though there is an upfront major equipment cost (for the luminometer). Finally, the assay does not require a non-N-fixing reference plant.

Fixed Nitrogen Assimilation and Translocation in Legumes

Nitrogen fixed by rhizobia inside legume nodules is secreted as ammonium into the host plant cell, where it rapidly assimilated
The GlnLux method is high-throughput, low cost, non-destructive, and there is no need for a reference plant. The GlnLux method can be used under controlled environmental conditions (laboratory/growth room/greenhouse) to measure SNF output with medium-high precision. However, SNF output measured by the GlnLux method is indirect, not time integrated, and may not precisely measure SNF output under field conditions. Symbol "✓" denotes the associated relative cost of the assay. The N balance method calculates SNF by considering all the N inputs and outputs on an area basis (kg N ha$^{-1}$). The N difference method calculates SNF based on the difference in shoot N accumulated by N$_2$-fixing plants vs. neighboring non-N$_2$-fixing plants, either a single plant or on an area basis. The ureide method is based on the % ureide N in the xylem sap or stem segments. The acetylene reduction assay is based on the activity of nitrogenase enzyme, where the reduction of acetylene to ethylene by nitrogenase provides an estimate of N$_2$ fixation activity. The $^{15}$N natural abundance method uses the natural difference in $^{15}$N abundance between atmospheric N and soil N to measure SNF, whereas the $^{15}$N dilution technique is based on the application of $^{15}$N-enriched N compounds to the soil. The $^{15}$N and ureide methods provide the percentage of N that is derived from SNF (%Ndfa). The amount of shoot N derived from SNF is calculated based on the shoot N content and %Ndfa (shoot N content x %Ndfa/100), on either a plant basis (mg N plant$^{-1}$) or area basis (kg N ha$^{-1}$).

### Various Factors Can Affect the Glutamine Concentration in Legumes

In general, the N transport form in xylem sap changes based on soil and environmental factors (rainfall/water availability, soil nutrient availability, salinity) (Neo and Layzell, 1997; Cramer et al., 2002; Amarante et al., 2006; Bai et al., 2007; Ladrera et al., 2007; Gil-Quintana et al., 2012, 2013). The experiments in this study were undertaken under optimal, controlled conditions with minimal external N. It is important to note that external N (e.g., soil N) is also assimilated into Gln, which has the potential to confound interpretations of GlnLux data with respect to SNF. For example, in soybean, a ureide exporter, exposure to NH$_3$ was shown to result in increased phloem Gln (Neo and Layzell, 1997) whereas another study showed similar xylem Gln levels when plants were supplied with NO$_3$ (20 mM KNO$_3$) compared...
to non-fertilized plants (McClure et al., 1979). A higher Asn:Gln ratio was observed in eight ureide exporters exposed to NO3, shown to be as a result of decreased Gln relative to Asn in cowpea (Pate et al., 1980). Xylem sap Gln was shown to change less significantly in amide exporting legumes in response to the source of N (SNF-dependent or external N) compared to ureide exporting legumes (Atkins et al., 1979; Amarante et al., 2006), likely because there is a larger relative background pool of Gln in the former.

In addition to external N, drought stress reduces SNF in legumes (Zahran, 1999; Giller, 2001; Kunert et al., 2016). Generally ureide exporting legumes (soybean, common bean) were shown to be more sensitive to drought stress compared to amide exporters (lentil, lupin) (Arrese-Igor et al., 2011). Therefore, the GlnLux assay may work more reliably with amide exporters than ureide exporters under drought stress conditions. Drought stress was shown to change Gln concentrations in amide exporters (Gil-Quintana et al., 2012) and ureide exporters (Silvente et al., 2012). The alteration in Gln concentration under drought stress varied by plant tissue (leaf/stem/root/nodule) (Ladrega et al., 2007; Gil-Quintana et al., 2012, 2013). It was found that under drought stress, leaf Gln content either decreased or remained close to the control (well-watered plants) in Medicago truncatula (amide exporter) (Gil-Quintana et al., 2012) and soybean (Silvente et al., 2012). Reallocation of leaf N compounds in legumes was shown to occur under drought conditions due to up-regulation of Gln synthetase (Aranjuelo et al., 2011). Further accumulation of total amino acids (Gil-Quintana et al., 2012) and proline during drought stress is a common scenario, where proline has been shown to act as an osmoregulant (Aranjuelo et al., 2011).

In addition, stress conditions that impair SNF can change the Gln concentration in xylem sap. For example, xylem Gln concentrations were shown to be reduced under water logging conditions in both amide and ureide exporting legumes (Amarante et al., 2006; Amarante and Sodek, 2006; Justino and Sodek, 2013). Interestingly, a similar pattern of reduction in xylem Gln was observed in both amide exporting (P. sativum, L. albus, C. juncea) and ureide exporting legumes (G. max, Phaseolus vulgaris, V. unguiculata) under water logging conditions similar to the reduction in SNF activity (Amarante et al., 2006). Therefore, in this study, Gln measurements were shown to be a good indicator of reductions in SNF under waterlogging stress.

Finally, Gln concentration can vary during the growth stage of legume plants. For example, Pate et al. (1980) found that the Asn:Gln ratio increased as cowpea and mung bean plants matured. However, the study did not show whether changes in the Asn:Gln ratio were due to decreased Gln or increased Asn.

### Limitations and Future Applications

The GlnLux leaf punch assay does not provide a direct measurement of SNF (%Ndfa) compared to 15N-based methods (Table 1). Another disadvantage is that GlnLux reports only the relative amount of Gln, not absolute amount. However, a concentration gradient of pure Gln standards can be used along with the leaf punch assay (Supplementary Figure S1), which was highly replicable. Although we found very strong correlation between GlnLux and 15N-based %Ndfa, the GlnLux method is a temporal snapshot and does not provide an integrated measurement of accumulated SNF output over time (Table 1). When collecting samples, leaf punches have to be flash-frozen in liquid N and kept frozen until processing, which limits its applicability to field conditions. Finally, as noted above, stress conditions such as drought can alter the leaf Gln concentration in legumes, which challenges the use of GlnLux to infer SNF under unpredictable conditions. Given this caveat, and since the leaf punch method cannot distinguish between N derived from SNF versus soil (Table 1), it may be most suited for pre-screening rhizobia inoculants under controlled N supply conditions prior to field experiments. We also expect that the GlnLux leaf punch method will assist with basic research especially to understand the unsolved mechanisms involved in the maintenance of SNF by facilitating high-throughput bacteria/plant mutant screens.

### AUTHOR CONTRIBUTIONS

Both MT and MR conceived of the manuscript. MT and MR designed the study and MT and NM conducted all experiments. MT wrote the manuscript and MR edited the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.01714/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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