SYMBIOTIC NITROGEN FIXATION IN DRY BEAN (*PHASEOLUS VULGARIS* L.) CULTIVARS

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Dry Beans (*Phaseolus vulgaris* L.), is the second-most important grain legume, and North Dakota has ranked first in dry bean production. The overarching research question of my project is whether we can increase the nitrogen (N) fixing potential of dry bean cultivars with inoculation and compare the nitrogen (N) fixing potential of common dry bean cultivars. Field experiment was conducted to compare peat and liquid inoculants during 2016 and 2017 growing seasons. In the field study, N_2 fixation was estimated by stable isotope (^{15}N) dilution technique. Liquid inoculant (70.5±5.9 kg ha^{-1}) showed similar potential to the peat based inoculant (60.5±4.7 kg ha^{-1}) for N_2 fixation, however, inoculation treatments did not increase the N_2 fixation over uninoculated (control). Further, it was investigated the *nifH* gene (marker for N_2 fixation) expression in the dry bean-*Rhizobium phaseoli* symbiotic system. It was found that the relative normalized *nifH* gene expression significantly correlates (r = 0.82) with the total amount of N_2 fixed, indicating the genetic control of symbiotic efficiency. Study on N-assimilatory genes, *NR* for nitrate reductase and *GS* for glutamine synthetase, showed that N_2 fixation alone could not support plant N need in the later stages of growth (i.e. late flowering) and supplementary application of mineral-N is necessary for better plant growth and economic-yield. Dry bean cultivars did not response to inoculation and genetically, they differed significantly in N_2 fixation potentials.

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DEDICATION

I dedicate my disquisition to my family, my parents Dipa and Deb Narayan, and My brother Dhiman.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

CHAPTER I: GENERAL INTRODUCTION

Nitrogen (N) or 'azote' (means without life) is an integral part of amino acids that make proteins (Zahran, 1999). Plants need N for their optimal growth and metabolism; but the available forms of N in soil, nitrate (NO_3^-) and ammonium (NH_4^+), which can be absorbed by the plants, are deficient in most of the soil under agroecosystems (Novoa and Loomis, 1981). Therefore, dependency on chemical fertilizers in crop production is increasing exponentially, and the cost of fertilizers input has also increased in last few decades (Lassaletta et al., 2014). Symbiotic nitrogen fixation (SNF), a biochemical process carried out by a specific class of bacteria (*Rhizobium*) in symbiosis with a specific class of plants (legumes), can add significant amount of N_2 into the global agro-ecosystems and reduce the use of chemical N fertilizers.

The atmosphere contains about 10^{15} tons of dinitrogen (N_2) gas, but this gas is highly inert and can only be transformed into usable forms of N by 'diazotrophs', a class of prokaryotes, mostly Bacteria and Archaea (Postgate, 1982; Ludden, 2001). These diazotrophs can fix N_2 gas as free-living organisms or symbiotically with legumes. *Rhizobium*, a genus of bacteria, can colonize legumes and fix N_2, which can be utilized in plant N metabolism (Hong et al., 2012). Population of *Rhizobia* in soils can vary depending on environmental factors such as climate, soil physicochemical parameters, and biotic-abiotic stresses (Singleton et al., 1991; Farid et al., 2017). Inoculation of legume crops with *Rhizobium* ensures symbiosis and increases probability of increased nodulation and subsequent SNF in the agricultural systems (Samudin and Kuswantoro, 2018).

Dry bean (*Phaseolus vulgaris* L.), the second-most important grain legume after soybean [*Glycine max* L. (Merrill)], is a great source of protein, fiber, and nutrition (Yonts et al., 2018). USA ranks fourth among dry bean producing countries in the world and North Dakota

(ND) ranks first among dry bean producing states in the USA (National Agricultural Statistics Service, 2016). Dry bean is a major crop in the Red River Valley (RRV) and ND shared 39% of total area in USA under dry bean production in 2016 (USDA-ERS, 2016). Dry beans add about 6 $\times 10^5$ Mg N annually in the agricultural systems worldwide, but fixes less amount of N_2 compared to other grain legumes such as soybeans (Farid and Navabi, 2015). *Rhizobium* inoculation in dry bean production is not popular in ND and only 14% dry bean growers in ND use inoculation in their fields (Knodel et al., 2017). Along with non-significant yield responses with *Rhizobium* inoculation, the commercially available peat-based inoculant for dry bean plugs the precision air seeder mostly used for planting in the RRV, which discourages the adoption of inoculation in the region. In this project, a new liquid inoculant was used as seed inoculation in the greenhouse and field experiments in addition to the common peat-based inoculant for dry beans.

In this project, determining the SNF potential of dry bean cultivars of two common market classes was the main goal. Use of stable isotope techniques is the most reliable approach to quantify the N_2 fixation, and these techniques have been reviewed extensively in earlier literature (Witty et al., 1988; Unkovich et al., 2008). Among all the stable techniques, isotope dilution was reported as the most efficient technique in quantifying SNF, but this method is costly and requires an IRMS (isotope ratio mass spectrometry) to analyze samples.

On the other hand, the ureide method is an alternative to stable isotope techniques to determine SNF activities in legumes under the Phaseolae and Desmodiae tribes such as soybean and dry bean, which export ureides (allantoin and allantoic acid) during SNF (Sprent, 2001; Coleto et al., 2014). Studies reported that ureide concentration in xylem sap when measured spectrophotometrically could be used as a measure of SNF successfully, and showed linear

correlation with the symbiotic dependence of the legume (Herridge, 1982; Goos et al., 2015). In this project, these two methods were first compared in a greenhouse study to choose between these two methods to estimate SNF in dry bean cultivars in our subsequent studies.

The *nifH* gene, a marker for biological nitrogen fixation (BNF), encodes nitrogenase enzyme responsible for N_2 fixation and is highly conserved in diazotrophs (Farnelid et al., 2011). Expression of this gene was studied in different environments (Mehta et al., 2003; Turk-Kubo et al., 2012; Warshan et al., 2016), but not in a symbiotic system. Bürgmann et al. (2003) studied *nifH* gene expression in free-living diazotrophs in soil and found linear correlation between the gene expression and amount of N_2 fixed. In this project, expressions of *nifH* gene and the genes that encode nitrate reductase (*NR*) and glutamine synthetase (*GS*) enzymes involved in N assimilation were studied in *Rhizobium*-dry bean symbiotic system.

Our study was designed to determine the effectiveness of rhizobia inoculation in increasing SNF with molecular evidence of *nifH* gene expression in the dry bean-rhizobia symbiotic system. This dissertation is comprised of three objectives, presented in three chapters: first, compare the efficiency of ureide method with stable isotope dilution technique in estimating SNF; second, determine the N_2 fixing potential of commercial dry bean cultivars under field condition, third, the expression dynamics of N_2 fixing (*nifH*) and N-assimilatory genes to find the correlation between the genotypic expression and biological function.

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CHAPTER II: LITERATURE REVIEW

Dry bean (*Phaseolus vulgaris* L.) is an important food crop due its high contents of proteins, vitamins, phosphorus. According to a report by ICRISAT (2015), dry beans share largest area after soybeans, about 27 million ha during 2008-2010 globally. The production has increased at an annual rate of 1.9%, though the production had touched a lowest yield of 793 kg ha^{-1} during this period (Nedumaran et al., 2015). The low yields were mostly due to poor yield performance by developing countries that contributes almost 95 percent of global dry bean production. The disease intensity was also high in dry beans, which indicated that more research should be done on biotic-stress management (Beebe, 2006). Also there lies a gap between the potential yield of improved commercial varieties and actual yields by farmers which implies that input management is needed to narrow the gap (Graf et al., 1991). Almost 60 percent area under bean was accounted by four countries: India, Brazil, Myanmar and Mexico and produced 11 × 10^6 Mg of dry beans. India has the highest area under dry beans with 8.3 million ha that produces 3.4 million ton, whereas Brazil produces almost similar quantity in only 3.8 million hectares. USA had the highest yield of 2 tons per hectare, followed by China, Myanmar, Argentina and Indonesia producing more than 1 ton per hectare. A positive trends in production was found in Latin America, South Asia and South-East Asia. In Sub-Saharan Africa, the production has increaseed 4.4 percent during the period from 1994 to 2010, mostly due to more areas that came under dry bean cultivation; the yield increased only a little. The scenario is different in east Asia and developed countries. A fluctuating trend in production, decline in the area under cultivation and an increasing trend for yield was observed between 1990 and 2010. On an average, dry bean yield increased globally except for Africa and South and South-East Asia, but the area under cultivation is showing a declining trend worldwide. During 1994-2010,

the yield has increased at a rate of 0.9 percent annually in the developed countries, while the area under cultivation decreased at a rate of 1.6 percent annually. As a result, a reduction of eighty thousand tons in bean production was reported.

The composition of beans and the importance of dry beans are different in highest dry bean producing countries (Akibonde and Maredia, 2011). In India, Myanmar and Pakistan, *Vigna mungo*, *Vigna radiate* and *V. aconitifolius* are the major pulses; while in China, mung beans are the major type of beans. In Africa, Latin America and carribean region, *Phaseolus* is the major bean grown or may be the only bean grown.

According to a report by USDA-ERS (2016), the total area under dry edible bean cultivation in the United States was 714, 160 ha in 2015 and 672,590 ha in 2016 . The area under cultivation was down 4 percent in 2016 from the previous year. North Dakota alone shared 37 percent area in 2015 and 39 percent in 2016 of the total areas planted under dry beans in United States, followed by Michigan and Idaho in both the years. The US dry bean production was 1,363,360 metric tons in 2015 and 1,302,350 metric tons; the productivity was 1.97 metric tons per hectare in 2015 and 2.06 metric tons per hectare in 2016.

Among the dry bean market classes, Pinto bean was planted in maximum area under cultivation in US; 246,453 ha in 2014; 234,960 ha in 2015 and 251,593 ha in 2016, followed by Navy and Great Northern beans. Dry bean production in US during 2016 under different market classes also followed the similar trend. Pinto bean production was 539,728 tons, followed by Navy and Dark red kidney beans that produced 196,896 and 63056 tons respectively in 2016. The bean production decreased in all the market classes, significant decrease in production by 17.7% in 2016 from that of in 2014 (239,288 tons) was found in Navy beans. The highest dry bean productivity in 2016 was found in Great northern beans (2602 kg ha^{-1}), followed by light

red kideny (2577 kg ha^{-1}) and Dark red kidney beans (2456 kg ha^{-1}). The productivity in 2016 was higher than the previous years, except only for Navy beans that showed a slight decline in productivity. US also exports a large amount of dry bean produced in the country. In 2015, US exported almost 34% of total dry bean production. According to 2015 Bean Market Summary (USDA), US exported 463,299 netric tons of dry beans worldwide, mostly to Europe (134,086 metric tons) and Canada (101,179 metric tons) (National Agricultural Statistics Service (NASS), 2016).

Nitrogen metabolism in legumes

Plants can synthesize amino acids, subsequently proteins, from organic and inorganic sources of nitrogenous compounds. The most abundant source of N is the atmosphere, but the inertness of N_2 restricts its direct uptake and assimilation into amino acids. Legume plants can take part in symbiosis and can convert the dinitrogen into simple nitrogenous compounds like ammonia that can further be utilized to synthesize more complex molecules like glutamine through a process known as assimilation (Cherkasov et al., 2015).

Plants mostly uptake N as nitrate (NO_3^-) from the soil; otherwise they uptake ammonium (NH_4^+). Nitrate in plant body gets assimilated in a two-step process. First NO_3^- gets transformed into nitrite (NO_2^-) with the help of the enzyme nitrate reductase (NR 1.6.6.1) and nitrite reductase reduces the NO_2^- to NH_4^+. NH_4^+ is assimilated into amino acids and proteins are synthesized. Plants can also produce ammonium through other metabolic processes like photorespiration, amino acid catabolism or from symbiotic N_2 fixation (Cren and Hirel, 1999).

Nitrates are primarily assimilated in the roots and influenced by age and limited root growth due to space (Márquez et al., 2007). Nitrate reductase reduces the nitrates taken up by the plant to nitrite with pyridine nucleotide, which is highly reactive. Nitrites are then readily

transported to the chloroplasts in leaves and plastids in roots where it further reduced to NH_4^+ in a reaction catalyzed by nitrite reductase (Rosales et al., 2011).

N_2 fixation

Biological N fixation (BNF), the reduction of atmospheric dinitrogen (N_2) gas into bioavailable NH_4^+, constitutes an important source of nitrogen input in many natural ecosystems, both terrestrial and aquatic habitats (Wartiainen et al., 2008). It contributes around half of annual N inputs into the biosphere (Vitousek et al., 1997). The report of BNF was traced back to 1888 that involved fixation of N_2 by legumes (Bazhenova and Shilov, 1995). Later, this biochemical process was reported for many other prokaryotes and symbiotic systems.

In the center of this process lies an enzyme, nitrogenase, includes a family of proteins that reduce N_2 to ammonia (NH_3). The enzyme system consists of two proteins: i) 'dinitrogenase or nitrogenase' that binds the N_2, helps in its subsequent reduction and consists of Molybdenum (Mo) and Iron (Fe); and ii) Mo-dependent 'dinitrogenase or nitrogenase reductase' that reduces the dinitrogenase proteins (Burris and Robert, 1993). Einsle et al. (2002) described central unit structure of the FeMo-protein, but its reaction mechanism is still not yet been established completely (Hoffman et al., 2013; Li et al., 2013). It is believed that the Mo-center binds N_2 and reduces it (Yandulov and Schrock, 2003; Arashiba et al., 2013), recent researches discovered that the Fe-containing complexes can also catalyze the N_2 reduction (Anderson et al., 2013).

According to the most documented mechanism of dinitrogenase-based nitrogen fixation, ATP (adenosine triphosphate) hydrolysis transfers electrons to the dinitrogenase protein that helps in stepwise reduction of N_2 to NH_3. Two ATP molecules are needed to transfer one electron which accounts to at least 16 ATP per N_2 molecule fixed (Igarashi and Seefeldt, 2003). The reaction is given as:

$$N_2 + 8H^+ + 8e^- = 2NH_3 + H_2$$

It is evident from the above discussion that the process of N fixation requires energy to be consumed. Kammermeier et al. (1982) calculated the energy expenditure and found the values to be between 48-63 kJ mol^{-1}. Alberty (2001) did the energy calculation more precisely considering the apparent reduction potentials of biochemical reactions and the Gibb's free energy of ammonia formation was estimated to be between 63.2 and 180 kJ mol^{-1}.

Nodules are produced as the effect of symbiosis between legumes and symbiotic bacteria (rhizobia). The bacteria gets nutrient as carbonaceous substrates from the legume partner and supplies nitrogenous compounds produced from BNF back to the host- this beneficial effect is termed as 'partner fidelity feedback' and considered as an important factor for strong mutualistic relationship (Fujita et al., 2014). The root-nodule system consumes about 15-30% or more of the photosynthates produced by the plant that is used mainly for reduction of N_2 and subsequent assimilation of the NH_4^+ product, maintenance and growth of nodules, biosynthesis and transport of nitrogenous organic compounds (Minchin et al., 1981). However, recently fixed N_2 is used to meet the N demand of nodule, approximately the half of the N need is contributed from soluble pool of N (Pate et al., 1979).

The nitrogenous solutes produced from BNF are transported to other vegetative and reproductive parts via xylem and remain as soluble N pool (Atkins et al., 1980; Pate and Atkins, 1983). If N is required by the plant, these compounds are degraded to release NH_4^+ and other by-products and reassimilated as specific complement of amino acids to synthesize other needed metabolites and macromolecules. On the other hand, insoluble pool of nitrogenous products act as reserve N and mobilized whenever the N is needed. Macromolecules (like proteins) are fed

into soluble N pool for recycling through various processes like catabolism, reassimilation and resynthesis of nitrogenous metabolites (Lea and Miflin, 1980).

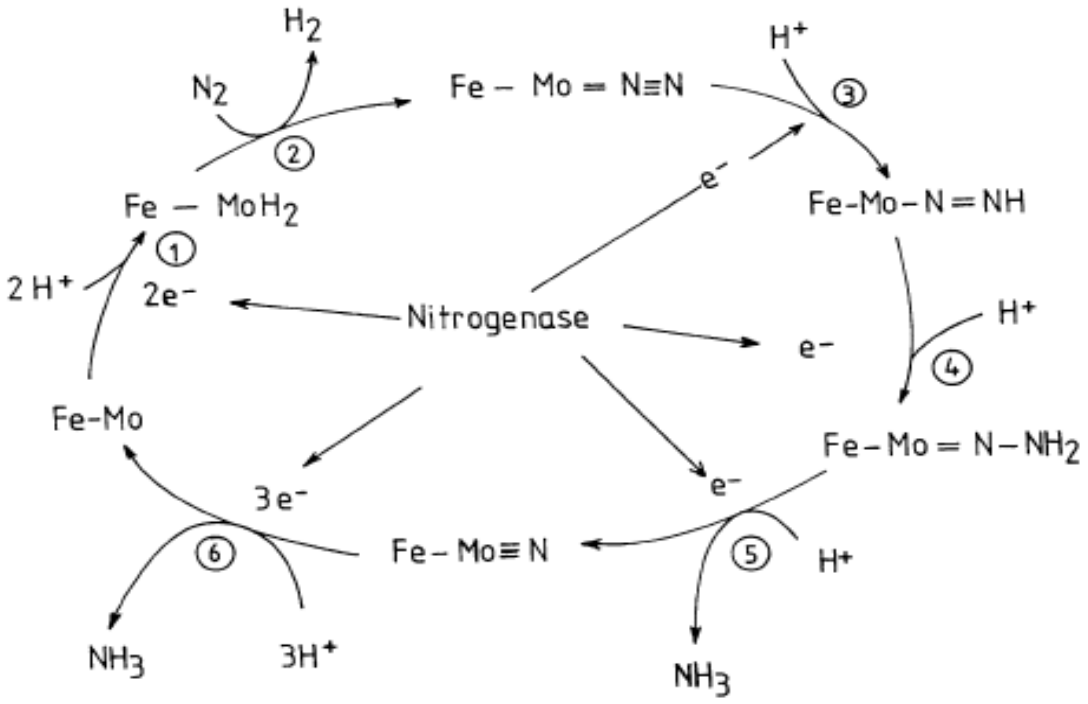

Figure 2.1: Biological Nitrogen Fixation: reaction sequence of reduction of N_2 to NH_3; compiled from Mengel et al. (2001)

Ammonium is the first stable product of BNF, released in the host cell cytoplasm in the nodules of leguminous plants and quickly assimilated into organic N compounds for transport (Bergersen, 1965). Depending on these compounds, the legumes can be classified into amide-exporters that transport asparagine (ASN), glutamine (GLN) or 4-methyleneglutamine (MeGln); or ureide transporters that transport N as allantoin (ALN), allantoic acid (ALC) or citrulline (CIT) (Schubert, 1986). The ureides are less soluble than the amides and found in higher amounts only in tropical legumes (Sprent and Embrapa, 1980). Patterson and Larue (1983) revealed that presence of nitrate and NH_4^+ in the rooting media influences a shift from ureide synthesis to amide synthesis, but the reverse process was not found.

N-assimilation

The inorganic N is first reduced to NH_3 before it can be assimilated as organic nitrogenous compounds in the plant system (Crawford and Arnst, 1993). Glutamine (GLN) and glutamate are the primary compounds in which the ammonia is assimilated in legumes. The N derived from the growth medium (like soil) through roots is mostly in the NO_3^- form, which is reduced to NH_4^+ in two-step process. The NO_3^- is first reduced to NO_2^- in the cytosol in a reaction catalyzed by plant nitrate reductase (NR) enzyme, depending on the NO_3^- and light availability (Solomonson and Barber, 1990; Campbell, 1999). The NO_2^- is then reduced to NH_4^+ with the help of the enzyme NR.

The major enzymes regulates NH_3 assimilation are glutamine synthetase (GS), glutamate synthase or glutaimine-2-oxoglutarate aminotransferase (GOGAT) and glutamate dehydrogenase (GDH) and present as isoenzymes encoded by distinct genes (Lam et al., 1996). Glutamine synthetase (GS; EC 6.3.1.2) is the key enzyme involved in the very first step of NH_4^+ assimilation, where it catalyzes the condensation of NH_4^+ and glutamate into glutamine with the help of another enzyme glutamate synthase in an ATP-dependent reaction (McGrath and Coruzzi, 1991; Cullimore and Bennett, 1992). There are three major sources of NH_3 in the plant and GS genes help plants to assimilate them into amino acids: NH_4^+ derived from soil solution in roots, NH_4^+ released through breakdown of the nitrogenous reserves in cotyledons during germination and NH_4^+ produced primarily from photorespiration. Mostly cytosolic GS regulates the assimilation of NH_4^+ in the roots and cotyledons, while the NH_4^+ produced from photorespiration is assimilated by the chloroplastic GS (Lea and Joy, 1983; Gebhardt et al., 1986). In N_2-fixing plants, GS in root nodules rapidly assimilates the NH_4^+ excreted into the

cytosol of the infected plant tissue before it reaches the toxic level (Atkins, 1987; Hardarson and Atkins, 2003).

There are two different types of glutamate synthase (GOGAT) in the plants: Fd-GOGAT that use ferredoxin as electron donors and NADH-GOGAT that use NADH as electron donors (Vanoni et al., 2005). Fd-GOGAT is mostly found in the chloroplasts, while NADH-GOGAT is present in the plastidic cells of non-photosynthetic tissues (like roots). The regulatory activities were extensively reviewed by Suzuki and Knaff (2005).

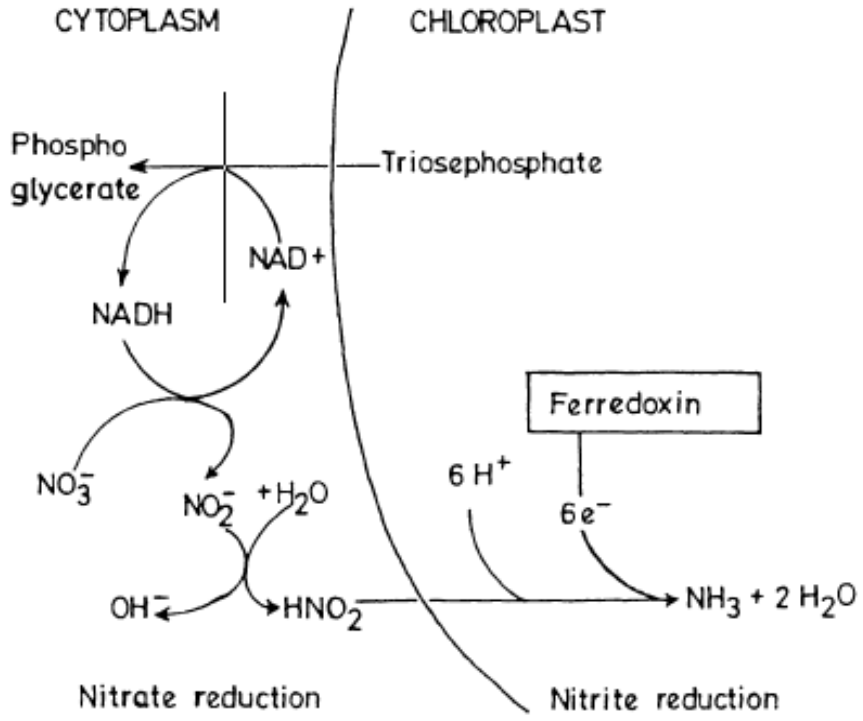

Figure 2.2: Nitrate and nitrite reduction; compiled from Mengel et al. (2001); NAD = Nicotinamide Adenine Dinucleotide

When Franco et al. (1979) studied N-assimilation in *Phaseolus vulgaris* L. plants, a relationship between nitrate assimilation and BNF was found. They reported that both the process benefited the plant, but maximum contribution of these two processes occur at different stages of growth. According to this report, peak nitrate reductase activity in plants followed by

maximal nitrogenase activity in common bean that differed from the phenomenon observed in soybean plants (Franco, 1977). Higher levels of NR activity in leaves after flowering was described as the ability of plants to absorb nitrates during this period. Addition of fertilizer N at flowering was also reported to be beneficial for common bean cultivars.

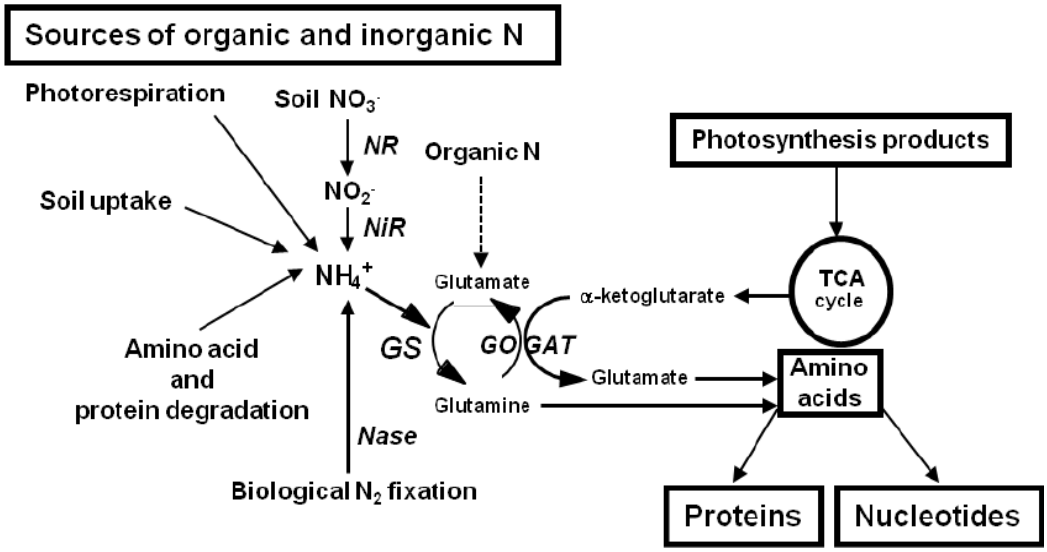

Figure 2.3: Nitrogen assimilation in legume plants; compiled from Hirel et al. (2011); NR: Nitrate reductase, NIR: nitrite reductase, TCA: tricarboxylic acid cycle

There was a large increase in the GS activities during nodulation along with the increased activity of nitrogenase when *Phaseolus vulgaris* L. was inoculated with *Rhizobium* (Lara et al., 1983). They stated that the increase in the activity is mostly due to a new form of enzyme in the root nodules, which was more or less similar to GS present in the roots. Robert and Wong (1986) analyzed GS isozymes in plant fractions in the root nodule extracts of 62 cultivars of common bean using polyacrylamide gel electrophoresis (PAGE). The result indicated that common beans generated isozymes of GS continuously during the nodule development. Robert (1989) reported that these multiple GS isozymes were not restricted to *Phaseolus* in a similar study in tropical legumes, *Vigna unguiculata* L. and *Glycine max* L. (Merrill) inoculated with *Bradyrhizobium* spp. and *Rhizobium fredii*.

Nitrogen demand for dry bean

Dry beans can fix atmospheric dinitrogen (N_2) symbiotically, but the relationship is not too strong and mineral N fertilization is often recommended for dry beans (Dow et al., 1973). With the efficient use of nitrogenous fertilizers, potential yield can be obtained. Dry bean cultivars vary in their ability of N_2 fixation, a starter N application is generally recommended as the N_2 fixed during early growth stages mostly utilized for nodule growth instead of plant growth (Sprent and Thomas, 1984). Brown and Westermann (2000) suggested that if dry bean crop follows a highly fertilized crop, generally N fertilization is not needed. Excessive N in the soil was reported to inhibit the nodulation, stimulate heavy vine growth, delay maturity, provide conditions favorable to insect activity, and enhance white mold and bacterial diseases. Dry beans are also grown following alfalfa, N fertilization is not needed as alfalfa residues release N that should fulfil dry bean N requirement. In a three-year long study, Gillard (2000) suggested that pre-plant or side banded N application increased yield in two of the years, but the yield increase was too small to pay for the cost of fertilizer N, but in the years with root rot stress, the yield response should increase. Where high amount of cereal residues are incorporated, fertilizer N should be applied either late fall after soil temperatures fall below 10°C or pre-plant in the spring as was shown for corn and sugar beets (Brown, 1988).

Dry bean cultivars differ in their response to N fertilization; cultivars with vigorous vegetative growth have higher N use efficiency as well as greater N fixation potential (Westermann et al., 1981). Early maturing cultivars were reported to be more responsive to the N fertilization than late-maturing varieties (Piha and Munns, 1987). The variability among the cultivar response due to N fertilization can be attributed to i) variable symbiotic efficiency; ii) dependence on starter N fertilizer; iii) variability in the amount and pattern of N requirement; iv)

variability in the availability of soil mineral N; v) lesser energy is needed to uptake mineral N than fix N_2 symbiotically (McKenzie et al., 2001).

Previous studies reported that dry beans can fix as much as 125 kg ha^{-1} (Rennie and Kemp, 1982, 1984). Under irrigated conditions, Davis and Brick (2014) recommended 70 kg N ha^{-1} for Colorado soils when inoculated for a target dry bean yield of 2800 kg ha^{-1}. They also suggested nitrogen rates for irrigated dry beans according to the concentration of ppm NO_3^--N in soil: 73 kg N ha^{-1} for 0-10 ppm, 50kg N ha^{-1} for 11-20 ppm, 28 kg N ha^{-1} for 21-30 ppm and no N fertilization in soils having more than 30 ppm nitrate N in soil. Long et al. 2010 stated that dry bean seed has 24 percent protein and 4 percent N on an average. They calculated the N demand by the crop in a practical way. According to their calculation, to get a yield of 2250 kg ha^{-1}, the dry bean crops need at least 90 kg N ha^{-1}. Also, the plants need 25 to 45 kg N per ha for its own growth. The plants can get about 20-40% of N through symbiotic N_2 fixation, but the plants should get the rest from other sources (like from soil or fertilizer). The calculation is most appropriate for the early maturing bush varieties that need a readily available source of N to achieve the potential yield. They also suggested that residual N levels in the soils (upto 24 inches) should also be accounted while calculating the side-dressed amount of fertilizer.

Fertilizer-N rates can reduced in the irrigated dry bean growing areas if the irrigation water contains dissolved nitrates. The following equation can be helpful in calculating added N from irrigation (Hergert and Schild, 2007)

N (lbs per foot of irrigation water) = ppm nitrate-N in irrigation water × 2.72

Soil NO_3^-–N content in combination with target or expected yield, previous crop and soil organic matter (SOM) content can be used as N fertilization guide. University of Minnesota

Extension came up with an equation for N recommendation using these parameters (Kaiser et al., 2011):

$$N_{rec} = 0.06\,EY - STN_{(0-24\,inches)} - N_{Pc}$$

EY = expected yield (kg ha^{-1}); STN = NO_3^--N measured to a depth of 61cm (kg ha^{-1}); N_{Pc} = amount of N supplied by the previous legume crop like alfalfa or red clover (kg ha^{-1}). The authors also mentioned that these suggestions were appropriate for non-irrigated fine textured soils, but might not be appropriate for irrigated sandy soils.

Under dryland conditions in North Dakota and Minnesota, 45 kg N ha^{-1} for inoculated crops and 80 kg ha^{-1} for uninoculated crops considering the soil test N were recommended by Franzen (2006). According to the report, due to dry, hot weather, short periods of saturated soil conditions and cold weather results in weak symbiosis between dry bean and rhizobia. It was also reported that fertilization at a rate more than the recommended dose did not benefit the crop yield, instead, increased disease and pest insurgence and plants matured late. The author concluded that no yield potential scale was reported due to the fact that the most economic N rate was not related to yield potential; when the environmental conditions were favorable, the crop yields were higher as the soil organic matter mineralization added more N to the plant available N pool in the soils.

Potential of *Rhizobium* inoculation to increase yield, N uptake and N use efficiency

Legumes (order Leguminosae) can only fix N_2 in a symbiotic association of specific bacteria genera like *Rhizobium* or *Bradyrhizobium* and their population in different environments may vary (Hellriegel and Wilfarth, 1888). Only a specific *Rhizobium* species can take part in the symbiosis with a specific legume species. The specificity of bacteria and legume species for effective symbiosis is known as cross-inoculation group (Lieberman et al., 1985). Lectins, sugar

binding proteins present in the cell wall of the root cell, play a major role in recognizing the specific bacteria and then, the infection process starts (Long and Ehrhardt, 1989). Some crops may be nodulated by a number of bacteria, but the nodules become ineffective due to non-specific infection (Mhamdi et al., 2002). Through inoculation, the specific rhizobia are supplied to the soil. Many soil and environmental factors influence symbiosis like temperature, moisture, redox status etc. and the success of inoculation also depends on these factors (Mohammadi et al., 2012).

Rhizobia population and strains vary spatially; soils with higher population of rhizobia do not need inoculation, but in soils with low population density, the additional supply of viable and effective rhizobia through inoculation can result in the successful symbiosis between legumes and rhizobia (Brockwell and Bottomley, 1995; Deaker et al., 2004). However, poor symbiosis or nodulation does not always lead to poor yield as the plant can take up N from soil reserve, which in turn exploits the soil-N reserve is not sustainable (Herridge et al., 1984).

Inoculation helps in higher N_2 fixation, but N_2 fixation does not always correlate well with the seed yield (Rennie and Kemp, 1982). There are variations among the crops, their cultivars, their symbiotic efficiency and their interaction with rhizobia strains. In addition, the amount of N_2 fixed varies depending on the method of determining the N_2 fixation or the crop used as reference. Bremer et al. (1988) reported that inoculation increased dry matter yield, total plant N and BNF of all the cultivars of grain legumes under study, though the proportion of N derived from the atmosphere declined with increasing soil nitrate levels and drought stress. This study also reported that indigenous rhizobia were incapable of supporting adequate N_2 fixation, which was a major factor influencing inoculation response. However, inoculation effect on N_2 fixation in the dryland condition was also influenced by moisture availability. Singh et al. (1998)

reported that pigeon pea (*Cajanus cajan*) responded favorably to N fertilization and *Rhizobium* inoculation for grain yield when grown in a soil with low organic carbon and available N and remained statistically similar when 0, 15 or 30 kg N ha^{-1} were added. Erman et al. (2009) showed that inoculation had significant effect on improvement in yield of field pea and the maximum yield was observed when 60 kg P_2O_5 per ha was applied along with the *Rhizobium* inoculation. Bengtsson (1991) found that inoculation and fertilizer N improved seed yield, protein content of common beans in the field where beans were never grown before. Nodule numbers decreased with N fertilization but improved significantly with inoculation. Denton et al. (2013) reported that inoculation increased nitrogen fixation in faba bean, but the increment was higher in the soils with low-N. Hynes et al. (1995) found that a liquid inoculant improved seed yields of soybean plants; and resulted in equal or better yield in pea and lentil than commercial peat based inoculants. Addition of cover inoculation with *Bradyrhizobium* in soybean during sowing or at V_3 stage improved seed yield by 50 to 90 g m^{-2} as revealed in a study by Ciafardini and Lombardo (1991), but in one-month delayed sown soybeans yield increment was absent. Nleya et al. (2001) observed the response of common ban to a granular inoculant and concluded that inoculation resulted in greater nodule mass, fixed N_2, and seed yield. Variable responses were found depending on the dry bean cultivars with percent N derived from atmosphere (%Ndfa) values ranged from 51 percent to 78 percent. They also reported that side-banded application of inoculation resulted in delayed maturity, which can potentially limit the feasibility of this treatment when early maturity is a required trait. Sogut (2006) showed that inoculation improved plant N content, dry matter, and seed yield compared to a fertilizer dose of 75 kg N ha^{-1} in soybeans. His study also indicated that inoculation was more effective in late-maturing cultivars. Abi-Ghanem et al. (2011) studied the inoculation effect of commercially available strains of

rhizobia on lentils and peas. This study reported that percent of N_2 fixed was significantly correlated with number of nodules and crop variety in both lentils and peas. The authors suggested that developing varieties with high rhizobial infection rates could be a rational strategy in enhancing crop N_2 fixation. In addition, rhizobia strains affected differentially to N_2 fixation in lentils but not in peas. Rudresh et al. (2005) tested the effect of combined inoculation with *Rhizobium*, *Trichoderma* spp. and phosphate solubilizing bacteria (PSB) in chickpea for growth, nutrient use, and yield of chickpea (*Cicer arietinum*). The study revealed that this combined inoculation improved germination, nutrient uptake, vegetative growth, nodulation, dry matter and pea yield than individual inoculation and control. They also concluded that no significant inhibition was found between the introduced organisms. Afzal et al. (2010) showed that co-inoculation with *Bradyrhizobium* and PSB increased the grain yield by 38 percent in the pot experiments and 12 percent in the field experiments. Survival efficiency was upto 48 percent higher for *Bradyrhizobium* when inoculated with PSB compared to its individual inoculation. Wang et al. (2011) reported complementary relationship between root architecture and Arbuscular Mycorrhizal Fungi (AMF) colonization under low P status in the soil. This study indicated that co inoculation with rhizobia and AMF improved soybean dry matter and root architecture under low N and low P conditions that helped better N use efficiency.

More efficient of *Rhizobium* strains have been selected for use in crops, less effort was given to select the lines. Pereira et al. (2015) investigated common bean lines for their interaction with *Rhizobium* and ability to utilize N fertilization. They conducted eight field trials in the Brazilian savanna using 17 ‘carioca’ elite lines. The authors reported that environmental factors influenced the symbiotic interaction and modified the elite lines’ responses to N fertilization, mostly when mineral N was added.

Commercially, inoculants are specific *Rhizobium* strains in a carrier material. Peat is the most commonly used carrier material for commercial inoculants of *Rhizobium* spp., but is not available in suitable forms in many countries (Halliday and Graham, 1978). Other carrier materials include peanut-hulls, ground corn-cob, hardwood charcoal or extender polymers (Sparrow and Ham, 1983). Survival of the bacteria depends on these carrier materials. A synthetic material is advantageous over natural substances due to less variability (Dommergues et al., 1979). The quality parameters of inoculants like number of rhizobia delivered to seed is essential as quality check procedures vary with countries (Thompson, 1980; Lupwayi et al., 2000). Concentration of commercial inoculum is also an important factor for optimum N_2 fixation in different soil moisture conditions (Dean and Clark, 1977).

Wolf et al. (1982) concluded that at least ten million viable rhizobial cells per gram of peat inoculant could be considered as good quality inoculant, though it varies with the condition. Singleton and Tavares (1986) reported that native *Rhizobium* population were effective in limiting nodule dry weight as well as N_2 fixation when the indigenous rhizobia population was over certain threshold level. (Singleton et al., 1991) studied the effect of indigenous *Rhizobium* population size on the symbiotic performance of field-grown legumes under inoculation. They studied seven legume species namely soybean (*Glycine max*), lima bean (*Phaseolus lunatus*), cowpea (*Vigna unguiculata*), bush bean (*Phaseolus vulgaris*), peanut (*Arachis hypogaea*), *Leucaena leucocephala*, tinga pea (*Lathyrus tingeatus*), alfalfa (*Medicago sativa*), and clover (*Trifolium repens*) inoculated with three effective strains of homologous *rhizobia*. It was found that soybean responded more efficiently to inoculation, while cowpea failed to respond in all the trials. Lack of inoculation reported even when most of the nodules harbored the inoculation strains that indicated a necessary improvement of inoculation technology.

McKenzie et al. (2001) showed that *Rhizobia* inoculation was unable to increase seed yield and did not reduce plants response to added fertilizer N. However, inoculation improved early season plant growth. Native *Rhizobia* were reported to be more efficient in increasing seed yield and N_2 fixation for one cultivar under the study, but not for the other cultivar (Tajini et al., 2008). Evaluating *Rhizobium* viability in commercial inoculum, they also found that inoculation in one year was able to maintain *Rhizobia* population in the soil.

The main objective of farming is to get higher yield and earn more money. Inoculation does not always increase yield which is a major factor that farmers are not interested in it (Graham, 1981; Sparrow and Ham, 1983). Sometimes the seed inoculation is not feasible with the planters or other planting instruments (like air-seeder). Sometimes methods are not efficient enough to supply large number of viable bacteria (Gemmell et al., 2002).

Use of stable isotope signatures in understanding legume N metabolism

Stable isotopes are elements with different numbers of neutrons in their atoms, but they do not show radioactive properties. There are two isotopes of N, ^{14}N and ^{15}N, the lighter one (^{14}N) is dominant in occurrence in the environment naturally. The ^{15}N occurs in the atmospheric air as $^{15}N_2$ at a more or less constant abundance of 0.3663 atom % (Mariotti et al., 1983). In order to investigate the N metabolism and distribution in higher plants, scientists started using ^{15}N isotopes. Emission spectrometer was used initially to determine the abundance of ^{15}N but only for small-size samples such as separating individual amino acids with thin-layer chromatography (Yoneyama and Kumazawa, 1972). Subsequently, the use of Isotope-Ratio Mass Spectrometers (IRMS) became popular to analyze ^{15}N abundance at low-enrichment and natural abundance levels precisely and more efficiently (Yoneyama et al., 2003).

The percent N derived from atmosphere (%Ndfa) or SNF by legumes can be precisely quantified under field condition only using the ^{15}N stable isotope technique (Rennie et al., 1982). The isotope dilution technique efficiently discriminate the N derived from atmosphere through fixation and the plant available N from soil or other growing medium when an appropriate non-fixing reference plant is used in the study (Danso, 1986; Barrie, 1991a) as the estimate vary depending on the non-fixing control crop (Wagner and Zapata, 1982)). The technique is most appropriate if the %Ndfa value is more than 70 percent, but less efficient for %Ndfa values less than 30 percent (Hardarson, 1985). McAuliffe et al. (1958) used the 'Isotope dilution technique' to estimate the SNF by legume (Ladino clover) grown in association with a grass (fescue), used as a non-fixing reference plants for the first time. The major assumption was that the legume under study and the non-fixing reference plants should extract isotopically identical N from the growing medium. However, studies reported that plants of different species vary in their root growth, rate and timing of N uptake (Boller and Nösberger, 1988; Peoples et al., 1989) and as a result, there will be dilution effect triggered by mineralization of non-labelled soil N (Witty, 1983). To avoid these limitations, several precautions like use of more than one reference plant, monitoring the chemical form and the amount of ^{15}N fertilizer applied, choosing the type and time of fertilizer application were reported (Reiter et al., 2002).

In a two-year long study, Rennie (1986) compared soil ^{15}N labelling technique and found out that post-emergence injection of ^{15}N tagged fertilizer in the rooting zone improves experimental precision more than pre-emergence broadcast or banded application of liquid ^{15}N avoiding loss of fertilizers during the spring. The use of ^{15}N natural abundance method in soils with more than 7 per mille $\delta^{15}N$ content provide better N_2 fixation estimates as they are more 'operationally uniformly' labelled with ^{15}N. Variations in the natural abundance of ^{15}N in the

plant tissues are good indicators of plant N uptake as natural substances have specific isotope signatures (Handley and Raven, 1992). Nodulated legumes showed negative $\delta^{15}N$ value, which indicated significant fractionation of ^{15}N over ^{14}N during N_2 fixation (Yoneyama, 1987). $\delta^{15}N$ values of soybean and cowpeas were found variable, sometimes up to +8.8 per mille, first observed only in ureide-exporting nodules or actively N_2 fixing nodules and the highest enrichment was found in the bacteroids (Shearer et al., 1982). Later, a study on asparagine-exporting pea and broad bean reported values between +0.9 per mille and +10.4 per mille in the nodule cytosol (Yoneyama, 1988). Unkovich et al. (1994) studied clovers, pea, and lupin under south-west Australian condition to determine the precision of estimating %Ndfa. They suggested that estimates of %Ndfa were erroneous when is correct $\delta^{15}N$ values (or B-values) were chosen. They also predicted the precisions of %Ndfa estimations for situations ranging from where a legume obtained 10 to 90 percent of its total N from SNF using $\delta^{15}N$ values of reference plants in the same study, and concluded that very high $\delta^{15}N$ values in reference plants when the legume is minimally dependent on fixation. Chalk (1996) concluded that there was no evidence that 'A-value' approach of estimating N_2 fixation was more precise or accurate than classical methods like ^{15}N dilution method. On a contrary, erroneous results were predicted when the symbiotic dependency of the plants was low. Authors suggested that modification of simpler classical techniques with A-value approach is not justified.

The non-fixing reference plants are of much importance estimating the N_2 fixation by legume plants using isotope dilution technique. Rennie et al. (1982) reported that uninoculated plants of nodulating legumes or legumes inoculated with an ineffective strain of *Rhizobia* were best to be used as reference plants in an isotope dilution study. The non-nodulating isogenic lines reported to overestimate the %Ndfa (percent nitrogen derived from atmosphere) values and

considered as an improper reference plant while studying soybean plants. Barley (*Hordeum vulgare*) was also found to be useful as reference plant. Bremer et al. (1988) suggested that wheat (*Triticum aestivum* L.) also performed similarly as a reference plant in N_2 fixation studies.

Methods involving ^{15}N were the most recommendable techniques to understand N dynamics in the plant system, although no method is precise as they are influenced by several factors that influence their efficiencies (Valles et al., 2003). Initial stable isotope studies involving $^{15}N_2$ suggested that NH_4^+ was assimilated by the reaction catalyzed by an enzyme glutamate dehydrogenase, but a study on soybean using $^{13}N_2$, a short lived radioisotope of N, proved that NH_4^+ is first attached to the amide position of glutamine synthetase (Meeks et al., 1978). When fed with $^{15}NO_3^-$ from petioles in soybean leaves, results from SIMS (Secondary Ion Mass Spectrometry) analysis suggested that first transported to epidermis and then to bundle sheath and mesophyll cells by symplasmic transport for the synthesis of amino acids (Grigron et al., 1999). Biological effects and toxicological hazards of chemicals and contaminated samples on plant metabolism were assessed by a stable isotope method known as ESIMA (Ecotoxicological Stable Isotope Metabolic Assay) using ^{15}N stable isotope in epicotyl tissues of *Pisum arvense* (Jung et al., 1999). The test is reproducible and very sensitive, hence can be a promising toxicity indicator test.

The primary organic compounds produced from N assimilation pathways are glutamine (GLN) and glutamate (GLU). A study reported that when $^{15}NO_3^-$ was supplied to the plant leaves, GLN and GLU, NH_4^+, aspartate (ASP) and asparagine (ASN) were produced as main products (Yoneyama et al., 2003). Labelling of GLN was found to be stronger and quicker than GLU, which indicates that GLU is produced from GLN (Thorpe et al., 1989). Respiration provides carbon skeleton for GLU synthesis, NADH for the nitrate reductase (NR) and ATP for

Glutamine synthetase. Relative ^{13}C-depletion of glutamate compared to asparagine that originated from oxaloacetate (OAA) synthesized from phosphoenolpyruvate carboxylase (PEPC) activity was reported in another study (Tcherkez and Farquhar, 2006). The report suggested that during GLU synthesis, 2-oxoglutarate molecules were provided via Krebs cycle, as earlier proposed by Gálvez et al. (1999). Yoneyama and Ishizuka (1982) studied soybean plants for primary distribution of N using the $^{15}NH_4^+$ and $^{15}NO_3^-$ in culture solution and $^{15}N_2$ from atmosphere. They reported that $^{15}N_2$ was fixed in the root nodules and quickly transported to the developing leaves and petioles; while the NH_4^+ was retained in the roots after its absorption and later transported to the developing leaves and petioles; $^{15}NO_3^-$ was distributed in a similar way. They concluded that the difference in the distribution in the soybean plants might be due to the differences in the major forms of ^{15}N transported in the xylem. Ureides that produced in the nodules containing the fixed N_2, were the major N-transporting forms in N_2 fixing soybean plants. Nitrates were mostly transported to the shoot as nitrate and asparagine; and ammonium as glutamine and asparagine. These results were confirmed as ^{15}N-labelled asparagine, allantoin and NO_3^- were fed to detached shoots at the vegetative and pod-filling stage. It was found that 85 percent and 88 percent of the plant fixed N_2 was distributed in the plants during vegetative stage and grain-filling stage respectively, the rest amount remained in the nodules where it incorporated in insoluble N compounds. The study also revealed that the fixed N_2 in roots were mostly recycled while the root protein N fraction that originated from the NO_3^--N came through two different processes; either through direct assimilation in the roots or through retransfer from the shoots. Pausch et al. (1996) used ^{15}N isotope to study effects of O_3 on N uptake, fixation and partitioning in soybeans. They found that exposure of plants to the O_3 at R_2 growth stage

decreased N_2 fixation and increased soil N uptake due to reduced photosynthate translocation to the nodules, but the total N and N% in organs were not affected.

Molecular tools to understand and improve legume N metabolism and inoculation response

The N_2 fixing microorganisms contain a special enzyme called nitrogenase that catalyzes the BNF, which has two components: MoFe protein (nitrogenase) and iron protein (nitrogenase reductase). Nitrogenase is a complex enzyme and composed of a heterotetrameric core encoded by *nifD* and *nifK* genes; while nitrogenase reductase is encoded by the *nifH* gene, a marker for biological N_2 fixation (Stacey et al. 1992; Peters et al., 1995) and used for studying the ecology and evolution of N_2 fixing bacteria (Raymond et al., 2004).

The *nifH* gene product (the polypeptide of the nitrogenase Fe protein) was found to have a molecular weight of 33,000 (Fuhrmann and Hennecke, 1984). The complete nucleotide sequence of the *nifH* gene was established, and the amino acid sequence of its gene product was deduced. The reading frame is 882 nucleotides long, corresponding to 294 amino acids, which add up to a polypeptide with a molecular weight of 31,525. There was extensive sequence homology with *nifH* genes or gene products from other N_2 fixing bacteria. Hong et al. (2012) did phylogenetic analysis of three *nifH* gene clusters in *Paenibacillus sabinae* T27 and revealed that all the three genes were expressed when N_2 fixation is going on.

It was reported that nitrogenase genes and 16S rRNA evolved parallel within a bacterium and subsequently proposed that *nifH*, *nifD* and *nifK* genes have evolved with the N_2 fixing bacteria harbored them (Hennecke et al., 1985). This theory argued that *nifH* genes might have evolved mostly depending on the bacterial evolution that carried them instead of horizontal *nif* genes transfer at the early stages of the prokaryotic evolution. Eardly et al. (1995) reported that *R. leguminoserum* 16S rRNA allele occurred in three distantly related group of strains, but could

not come up with a satisfactory explanation. However, they concluded that the theory of full or partial recombination of genes and their horizontal transfer can be a reasonable explanation, but additional genetic evidence is needed to explain the evolutionary relationships of symbiotic partners of nodulating plants. Phylogenetic study on Phaseolus symbionts revealed that symbiotic genes are closely related with each other irrespective of their 16S rRNA based classification (Laguerre et al., 2001). This study also supported the fact that lateral gene transfer across rhizobial species, sometimes across *Rhizobium* and *Sinorhizobium* genera, is important in diversification and in structuring natural rhizobia population. Another study on phylogeny and genetic diversity of native rhizobia nodulating common bean plants revealed that symbiotic genes originated from a common ancestor different from the core genome of the species and supported the theory of horizontal gene transfer (Aserse et al., 2012).

The detection of *nifH* amplified from mRNA indicates the presence of N_2 fixing bacteria as well as evidence of N_2 fixation in plants (Young, 1992). Another study proved that N_2-fixation is not a genetic trait for most of the uncultured bacteria (Rösch et al., 2002). A diverse group of bacteria can nodulate the common bean plants and currently classified under three species: *Rhizobium leguminoserum* biovar *phaseoli*, *R. etli* bv. *phaseoli* and *R. tropici* (Jordan, 1984; Martínez-Romero et al., 1991; Segovia et al., 1993). Furthermore, two new *Rhizobium* genomic species (Laguerre et al. 1993) and other unclassified genotypes (Pinero et al. 1988; Martinez-Romero et al. 1991) were isolated from the common bean nodules (Piñero et al., 1988; Martínez-Romero et al., 1991; Laguerre et al., 1993). It has become more and more evident that the plants can be nodulated by more than one *Rhizobium* species and symbiotic performance can no longer be used to differentiate between species. Variations in 16S rRNA of different *Rhizobium* species were studied using RFLP (Restriction Fragment Length polymorphism) analysis amplified by

PCR (Polymerase Chain reaction) technique and it was found that PCR-RFLP method can be used as a rapid tool to identify the root-nodule isolates and can detect new taxa of nodulating partners (Laguerre et al., 1994). Furthermore, it was also reported that a modified form of RNA polymerase was involved in recognizing the *nif* promoters (Ow et al., 1983).

The genetic diversity present in a microsymbiont population and factors other than indigenous soil rhizobia population like dominance of few specific bacterial strains play a major role in the selection symbiotic partner by common bean plants (Junier et al., 2014). A study on different cultivars of dry beans revealed the fact that the cultivar showing higher *nifH* gene expression also fixed high amount of N_2 from atmosphere (Akter et al. 2014). This study concluded that the *nifH* gene expression study could be a useful tool to select dry bean cultivars with high N_2 fixing potential.

Plant flavonoids induce *nod* genes in the bacteria that regulates the *nod* factor synthesis (Perret et al., 2000). *Nod* factors are the main nodulation signal molecules; type and quantity of *nod* factors determine the host specificity. Though rhizobia with different *nod* genes produce different *nod* factors can effectively nodulate the same plant as in case of *Rhizobium etli* bv. *phaseoli* and *Rhizobium tropici*; they both effectively nodulate common bean plants (Poupot et al., 1993). The production of flavonoid *nod* gene inducers is influenced by environmental factors like plant fertility, pH and *nod* factors (Schmidt et al., 1994). *nod*ABCD genes are found in all rhizobia and it was found that the *nod* genes were highly conserved among distantly related rhizobia when nod gene sequences were analyzed (Debellé et al., 2001). This result indicated that these genes might have monophyletic origin and through horizontal gene transfer processes, transmitted to non-symbiotic bacteria. The authors also stated that these nodulation genes regulates specific biosynthesis and secretion of lipo-chitooligosaccharides, also known as the nod

factors, which even at a very low concentration can influence the symbiotic responses of a host plant.

Most of the energy for NO_3^- assimilation was derived from the plant photosynthesis, which was postulated to be the reason behind the light regulation of *NR* activity and *NR* gene expression and supported by earlier researchers (Lillo et al., 1996; Provan and Lillo, 1999). The transcription of *NR* is regulated through one or more elements in the promoter as studies identified transduction mutants with abnormal *NR* transcription (Ogawa et al., 2000). NR enzyme activity and *NR* gene expression in cytoplasm was found to be controlled by the electron flow from photosynthesis (Sherameti et al., 2002). This study demonstrated that the *NR* gene was activated by the oxidized state of a component involved in electron transport.

Specific and quantitative abundance of mRNA related to cytosolic and chloroplastic *GS* cDNAs were studied using 'RNase protection technique' during nodulation (Bennett et al., 1989). They also reported the sequence of a full length *GS* gene clone (pc*GS*-γ1) isolated from a root-nodule cDNA library of dry bean. The researchers also suggested that the γ -polypeptide was actually a 'nodulin' protein, like leghemoglobin and other nodule-specific proteins, though the expression is not truly nodule-specific. They concluded that the kinetic properties of the encoded isozymes and their cellular and sub-cellular locations played key role in determining the physiological functions of the GS polypeptides. Roche et al. (1993) found that two different classes of the cytosolic *GS* genes were expressed in soybean nodules: one of them was nodule specific and only expressed in the nodules; but the other one was also expressed in roots, leaves and cotyledons (Roche et al., 1993). Nodule specific *GS* genes have been reported in other legumes as well and mostly studied in the dry bean, but it has also been reported that those nodule specific genes were expressed in other plant organs but at greatly reduced levels (Swarup

et al., 1990). Morey et al., (2002) investigated the cytosolic *GS* gene in soybean using 'Genomic Southern analysis' and identified three distinct classes of *GS* genes: α, β, γ. The authors suggested that the α forms were major isoforms in cotyledons and young roots, while the β forms were found to be nodule specific. The γ genes were divided into 2 subclasses; the γ_1 was more nodule specific whereas the γ_2, although nodule–enhanced, also expressed in cotyledons and flowers. Seabra et al. (2010) reported that *Medicago trancatula* contains an extra *GS* gene that encodes a plastid-located isozyme, was functional and expressed in developing seeds. To study the functionality of the genes, RT-PCR was done using specific primers, e*lf1-α* was used as a housekeeping genes for the study. They also concluded that this gene duplication might be exclusive to the legume seed metabolism related to storage protein accumulation. In another study (Seabra et al. 2013), the authors quantified individual *GS* transcripts using qRT-PCR (quantitative reverse transcriptase-polymerase chain reaction); and *GS* polypeptides and holoenzymes were evaluated by Western Blots and in-gel native electrophoresis (Seabra et al., 2013). They demonstrated that the *GS* gene expression are moderated during a diurnal cycle and the glutamine synthetase proteins were incorporated into complexes that assemble into a specific composition in the specific plant organs.

From the above discussion, it is evident that there are several aspects regarding SNF in dry bean that are not well explained. Three specific research objectives were hypothesized i) to compare the efficiency of ureide method and isotope dilution technique in determining SNF in dry bean, ii) to check if rhizobia inoculation can improve SNF in dry bean cultivars commercially grown in the northern great plains, and iii) to understand the expression dynamics of the genes related to SNF and N-assimilation and correlate the genotypic expression with the amount of N_2 fixed.

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CHAPTER III: DETERMINING SYMBIOTIC NITROGEN FIXATION IN COMMERCIAL DRY BEAN (*PHASEOLUS VULGARIS* L.) CULTIVARS USING UREIDE METHOD AND STABLE ISOTOPE DILUTION TECHNIQUE

Abstract

Symbiotic Nitrogen Fixation (SNF) is an environmentally-safe source of N to the crop plants. Twelve dry bean (*Phaseolus vulgaris* L.) cultivars from pinto, navy, black, and kidney market classes were inoculated with rhizobia and grown in a greenhouse. SNF was estimated using isotope dilution technique and 'ureide' method. Amount of SNF ranged between 33 and 68 mg N plant^{-1} when determined using ^{15}N isotope dilution and followed the order: pinto>navy>black>kidney. Percent N derived from atmosphere (%Ndfa) significantly varied between 49% and 90% at V_3, and between 71% and 98% at R_2 stage. Outcomes of the experiment suggested that dry bean cultivars from different market classes have variable N_2 fixing ability and fertilizer N required should be calculated according to their SNF potentials and N need of a specific market class or cultivar. Stable isotope dilution should be used as the standard procedure to estimate the SNF in dry bean.

Keywords: **S**ymbiotic Nitrogen Fixation; Dry Bean.; *Rhizobium*; ureide, isotope dilution; ^{15}N

Introduction

Symbiotic Nitrogen Fixation (SNF) has the potential to improve soil fertility, enhancing the nitrogen (N) balance in soil and N availability to the subsequent, or accompanying crops (Hardarson and Atkins, 2003; Triboi and Triboi-Blondel, 2014; Ronner et al., 2016). Globally, legumes can fix about 20×10^6 Mg of N per year to the agricultural systems (Herridge et al., 2008). *Rhizobium* spp. inoculations to leguminous crops are considered to increase N availability to the plant. However, legume cultivars respond variably to rhizobia inoculation, depending on

their symbiotic compatibility with the bacterial strain and environment conditions (Rennie and Kemp, 1982; Akter et al., 2014; Heilig et al., 2017; Youseif et al., 2017).

Dry bean, the most important food-legume crop, is the largest source of plant protein for direct global food-sufficiency (Tajini et al., 2008). Even though dry beans are poor N_2 fixer (fix 30-50 kg N ha^{-1}) as compared to other grain legumes (fix 80-100 kg N ha^{-1}), dry beans add about 6×10^5 Mg N annually in the agricultural systems globally (Kipe-Nolt et al., 1993; Herridge et al., 2008; Farid and Navabi, 2015). When inoculated with rhizobia, few dry bean cultivars were reported to fix N_2 (40-125 kg N ha^{-1}) equal to the amount that found in soybeans, but the inoculation effect is not consistent globally (Rennie, 1982; Rennie and Kemp, 1982). Reports of poor nodulation and lack of response to rhizobia inoculation in the field pose a serious concern regarding the benefits of inoculation in dry beans (Graham, 1981; Buttery et al., 1987). Along with environmental factors like extreme pH (both acidic and alkaline conditions), water stress and salinity, presence of native rhizobia in the soil is a major factor influencing the response of inoculation (Singleton & Tavares 1986; Thies et al. 1991; Farid et al. 2017). However, in dry bean growing areas of North Dakota and Minnesota, environmental stresses such as heavy rainfall and flooding, heavy wind, and biotic stresses such as weeds and disease such as white mold, bacterial blight, root rot severity, are the potential factors that limit nodulation and N_2 fixation (Knodel et al., 2017). Ultimately, the positive inoculation response depends on the genotype, genotype × *Rhizobia* strain interaction, inoculant formulation, and the application method (Thilakarathna and Raizada, 2017).

United States (USA) is the fourth–largest producer of dry beans, and produced 1.3×10^5 Mt of dry beans in 672, 590 ha of area in 2016 (FAOSTAT, 2014; USDA ERS 2016). Among the dry bean producing states in US, North Dakota is the largest producer of dry beans and alone

shared 39% of area under dry beans in 2016, followed by Michigan (13%), Minnesota (10%), and Nebraska (9%) (NASS, 2016). According to a grower's survey in 2016, only 14% dry bean growers in North Dakota use the rhizobia inoculants (Knodel et al., 2016). In addition to poor seed yield-response of the crop to rhizobia inoculation, the available common peat-based inoculants plug the precision air-seeder and increase planting time and labor (for seed inoculation). This discourages dry bean growers to adopt inoculation (Singleton et al., 2002). Liquid rhizobia inoculation resulted in comparable or better seed yields of soybean (*Glycine max* L. Merr.), field pea (*Pisum sativum* L.) and lentil (*Lens culinaris* Medik.) than peat-based inoculation (Ciafardini and Barbieri, 1984; Hynes et al., 1995). Liquid rhizobia inoculants for dry beans can be a potential alternative for peat-based or granular inoculants as seed treatment or through in-furrow application.

The use of ^{15}N, a stable isotope of N, is the most reliable and most accurate approach to quantify N_2 fixation and it has been extensively reviewed (Chalk, 1985; Witty et al., 1988). Among several ^{15}N-isotopic methods, the 'isotope dilution technique' has high efficiency in discriminating the N_2 fixed from the atmosphere and the N taken up from the soil, though this technique is most accurate when the 'percent N derived from atmosphere' %Ndfa exceeds 70% and less suitable when N_2 fixation values are less than 30% (Danso, 1986; Barrie, 1991). A non-fixing reference plant is needed to estimate %Ndfa and both the fixing and reference plants should uptake N from the soil with same ^{15}N isotopic composition (Boddey et al., 1984).

The 'ureide method' is an alternative method to estimate SNF only in legumes exporting ureides during N_2 fixation. Ureides (allantoin and allantoic acid) are synthesized from purine biosynthesis and purine oxidation pathways in the legumes within the Phaseolae and Desmodiae tribes such as soybean and dry bean (Sprent, 2001; Boldt and Zrenner, 2003; Zrenner et al.,

2006; Coleto et al., 2014). Studies on soybean and cowpea (*Vigna unguiculata* L.) have confirmed that these plants export fixed N_2 predominantly as ureides from the nodules to the shoot tissues through xylem stream (Matsumoto et al., 1977; Herridge et al., 1978). Concentration of ureides in the xylem-sap or aqueous extracts of dried plant tissues of these legumes can be used as a measure of N_2 fixation, irrespective of legume genotype or strains of rhizobia (Herridge and Peoples, 1990; Unkovich et al., 2008; Goos et al., 2015). Relative abundance of ureides with respect to nitrate in the plant shoots also showed linear relationships with the symbiotic dependence of the legume (Herridge, 1982).

Low N or N-free conditions force the plants to depend on seed and atmospheric–N_2 and can be used as a screening tool of cultivars for SNF (Rennie and Kemp, 1982; Miranda and Bliss, 1991). As genotypic variation exists for SNF in dry beans, cultivars should be screened to choose the most efficient genotypes for commercial and breeding purpose (Chaverra and Graham, 1992; Agoyi et al., 2017). We planned this study to identify the most efficient cultivars commonly grown in North Dakota based on their N_2 fixation potential as estimated through ureide method and isotope dilution techniques. Our objectives were i) to verify the ureide method in quantifying N_2 fixation in dry bean cultivars, ii) to determine the N_2 fixation potential of different dry bean cultivars within four popular dry bean market classes, and iii) to check the potential of the new liquid inoculant.

Materials and methods

Greenhouse study design and environment

A greenhouse study was conducted at North Dakota State University (NDSU) during spring 2016 to check the potential of the liquid inoculant and SNF of dry bean cultivars. The greenhouse temperature was maintained at 30˚-35˚C during day and 19˚-23˚C during the night.

Sixteen hours of light and eight hours of dark period was maintained in the greenhouse. Twelve cultivars, three from each of four dry bean market classes (pinto, navy, black, and kidney) were selected (Table 3.1). Cultivars were selected based on their higher acreage in North Dakota and Minnesota area as reported in 2014 Dry bean growers' survey (Knodel et al., 2015). Plants were grown in a sand-vermiculite (3:1 v/v) medium in 2500 cm^3 closed bottom pots and watered regularly to maintain water content at 50% water holding capacity (WHC). The pots were laid out in a completely randomized design (CRD) with factorial arrangement of twelve dry bean cultivars × two growth stages (V_3 and R_2) × five replications.

Planting and rhizobia inoculation

A liquid rhizobia inoculant (Excalibre SA™ for Dry bean, ABM™) was used in this study to supply specific group of rhizobia in the growth media. Dry bean seeds were washed with non-chlorinated water, and pinched for better germination in the greenhouse. Then, the seeds were soaked in the liquid inoculant containing *Rhizobium phaseoli* strain (rhizobia of specific cross-inoculation group for *P. vulgaris*) just before sowing and air-dried for five minutes. Four seeds were planted in each pot and one week after germination, plants were thinned to two plants per pot, and maintained.

Fertilization

Dry bean plants were fertilized with $K^{15}NO_3$ (enriched with 99.2 atom % ^{15}N) @ 1.75 mg N pot^{-1} in three split doses (7, 14 and 21 days after germination) for subsequent analysis following the isotope dilution technique. The plants were grown in N-stress initially assuming that it would force them to fix more atmospheric N_2. For other macro and micronutrients, the plants were supplied with N-free modified Hoagland Solution (Caisson Laboratories, Inc.) 200 ml pot^{-1} as basal dose, 100 ml pot^{-1} 7 days after germination, 70 ml pot^{-1} after 25 days after

germination (DAG) and 40 ml after 1st sampling at V_3 stage. However, to help them survive, plants were fertilized with NH_4NO_3 @ 25 mg N pot^{-1} after the second plant sampling at 45 days after planting (DAP).

Table 3.1: Description of 12 cultivars of drybeans selected in the study

Market Class	Cultivars	Origin	Dry bean plant type	PVP #
Pinto	La Paz	Provita	USV (II)	200500219
	Lariat	NDSU	USV (II)	200800305
	Windbreaker	Seminis	UV (IIb)	200500105
Navy	Medalist	Provita	USV (II)	200700330
	T9905	Hyland	USV (II)	N/A
	Ensign	ADM/Seedwest	UV (III)	9900273
Kidney	Red hawk	Mich. AES	B (I)	200000264
	Pink Panther	Seminis	B (I)	200300266
	Foxfire	ADM/Seedwest	B (I)	9200069
Black	Loreto	Provita	USV (II)	200700297
	Zorro	Mich. AES	USV (II)	201000268
	Eclipse	NDSU	USV (II)	200500293

UV: Upright Vine, USV: Upright short Vine, B: Bush; I: Bush- Determinate, II: Upright – Indeterminate; IIb: Upright indeterminate, but prostrated/ decumbent in some environments; III: prostrated/ Decumbent – Indeterminate; PVP #: Plant Variety Protection Number; Source: Drybean Production Guide, Edited by H. Kandel, NDSU Extension Service (2013)

Plant sampling and data collection

Plant samplings were done twice at third trifoliate or V_3 stage (35 DAP) and late flowering stage or R_2 (45 DAP). These two growth stages for sampling were selected as N_2-fixing activity is higher in these two stages of growth; V_3 is representative stage for vegetative growth and R_2 for reproductive growth (Franco et al., 1979). Both non-fixing and N_2 fixing legume plant samples were collected on the same day. Fresh plant samples were weighed, and then dried at 55-60˚C for 4 days. Only plant axis (stem and petioles) of one plant in each pot was used for ureide-N analysis, but for isotopic dilution measurements, the whole shoot (stem + petioles + leaves) of the other plant were used.

Ureide-N analysis

Dried plant axes were ground to pass a 0.1 mm screen. The plant tissue extracts were prepared by taking 0.2 g of ground-tissue into a screw-cap test tube with 20 mL of water. Then, the tubes were sealed and placed in water-bath at 90°C for 30 minutes. The tubes were then cooled down to the room temperature, shaken in a mechanical shaker for 30 minutes, and subsequently filtered to get clear extracts. Extracts were then stored in a refrigerator until analyzed for the ureide concentration.

The ureide concentration of dry bean shoot tissue was determined using the method proposed by Goos et al. (2015). The relative ureide-N (RUN) values were calculated using the following formula:

$$Relative\ Ureide - N\ (\%) = \frac{a \times 100}{(a + b)}$$

where, a and b are, respectively, the concentrations of ureide-N and nitrate in the plant axes (Herridge, 1982). Concentration of nitrate (NO_3^-) in the plants axes was determined using a rapid colorimetric (at 410 nm wavelength) method (Cataldo et al. 1975).

Isotope dilution technique

Dried plant shoot tissues were ground to pass 0.02 mm screen, and about 3 mg of ground samples was encapsulated into tin (Sn) capsules. Encapsulated samples were packed in 96-well trays and sent to UC Davis Stable Isotope facility. Samples were analyzed for isotopic ratio (i.e. ^{15}N: ^{14}N) using a continuous flow Isotope Ratio Mass Spectrometer (PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 IRMS). Percent N derived from atmosphere (%Ndfa) was used as an indicator of SNF and was calculated from 'atom% ^{15}N' values of plant samples using the following formula (McAuliffe et al., 1958):

$$\%\, Ndfa\ = \left(1 - \frac{atom\%\ 15_N\ excess\ in\ N_2 - fixing\ plant}{atom\%\ 15_N\ excess\ in\ reference\ plant}\right) \times 100$$

The 'atom % excess ^{15}N' is the ^{15}N enrichment above the background. i.e. atmosphere (the value is 0.3663%). A non-nodulating dry bean white navy mutant line, 'R99', was used as non-fixing reference (Park and Buttery, 2006). The ^{15}N atom% excess of these plants were used to calculate the %Ndfa values of the cultivars under study.

Statistical analyses

The data of plant dry matter, RUN, %Ndfa and N_2 fixed at the two sampling stages were analyzed for analysis of variance (ANOVA) in generalized linear model using 'Proc GLM' procedure in SAS 9.4 software (SAS Institute, 2013). For statistical analysis, all the factors, growth stages, market classes, cultivars nested within each market classes, and the interactions (growth stages × market class and growth stages × cultivars within market class) were considered as fixed factors. Least squared means were computed using LSMEANS statement, standard errors of least squared means were estimated, and pairwise significant difference tests were performed using the TUKEY statement at 95% significance level in SAS 9.4. Pearson correlation coefficients among plant dry matter, RUN, %Ndfa and N_2 fixed were also computed using 'correlation analysis' in SAS Enterprise Guide 6.1 software (SAS Institute, 2013).

Result and discussion

Plant dry matter

Plant dry matter varied significantly among the bean market classes, cultivars within market classes and between the two growth stages, V_3 and R_2 (Table 3.2). Among the dry bean market classes, navy (2.45 g plant^{-1} at V_3 and 2.48 g plant^{-1} at R_2) produced significantly lower plant dry matter than the other three market classes at both the growth stages (Table 3.2). Highest mean plant dry matter was produced by kidney (3.04 g plant^{-1}) at V_3 and pinto (3.01 g plant^{-1}) at

R_2 stage (Table 3.2). Pinto cultivars showed significant variation in plant dry matter production at V_3 stage, and 'Windbreaker' (2.86 g plant^{-1}) produced significantly higher plant dry matter than 'Lariat' (2.33 g plant^{-1}) and 'La Paz' (2.36 g plant^{-1}). Pinto market class had significant improvement in plant dry matter at R_2 (3.01 g plant^{-1}) stage from V_3 stage (2.52 g plant^{-1}). Among cultivars within pinto market class, 'Lariat' had significantly higher plant dry matter at R_2 (3.18 g plant^{-1}) than V_3 (2.33 g plant^{-1}) growth stage (Table 3.2).

All the cultivars produced lower plant dry matter than values reported earlier which was in the range between 4.67 and 7.73 g plant^{-1} (Andraus et al. 2016). One probable reason was low amount of N supplied from the growth media; also, weak symbiosis between the plant and the rhizobia could not fix enough atmospheric N_2 to support the N requirement of the plants for optimum vegetative growth. Nitrogen is indispensable for dry bean growth and dry matter production. The lack of N reduced the leaf growth and numbers, and in turn reduced the photosynthetic efficiency and overall plant growth.

Significant variation in plant dry matter among the dry bean market classes can be attributed to their differences in growth habit. Also, the N stored in the seeds might have played a vital role as N supply. As the total available N supply was very less, variation in the seed size and amount of N stored in the seeds could be a reason for variation in plant dry matter, as more N should produce higher plant dry matter. As the kidney and pinto market classes produce larger seeds, the plant dry matter contents were also found higher than black and navy beans at both the growth stages (Table 3.2).

Relative Ureide-N

The RUN values differed significantly among the market classes, cultivars within market classes, and between two growth stages (Table 2). Significant variation in RUN values were

found between dry bean market classes at V_3 stage. Kidney beans (66%) had significantly lower RUN values than the other three market classes, pinto (81%), navy (80%) and black (80%) at V_3 (Table 2). However, at R_2 stage, all the dry bean market classes had statistically similar RUN values. Black market class had the highest mean RUN values at both V_3 and R_2 (88%) stages, while pinto had the lowest mean RUN value at R_2 (84%). Except 'pinto', all the other market classes under study (i.e. navy, kidney and black) had significantly higher RUN values in R_2 than V_3 stage (Table 2).

The pinto cultivars showed significant variation in RUN values at both growth stages, while the kidney cultivars showed significant variation only at the V_3 stage (Table 2). Pinto cultivar 'La Paz' had significantly lower RUN values at both V_3 (64%) and R_2 (70%) stage than 'Lariat' (89% at V_3 and 92% at R_2) and 'Windbreaker' (88% at V_3 and 91% at R_2). Kidney cultivar 'Foxfire' (60%) had significantly lower RUN than 'Pink Panther' (69%) and 'Red hawk' (69%) at V_3 stage. Higher RUN values implied relatively higher amount of ureide-N concentration than NO_3^--N in the plant axes, and indicated higher fixed N_2 transport from nodules to shoots. Kidney cultivars had significant increment in RUN values at R_2 stage from V_3 stage; and the increments in RUN values were 42% 'Foxfire', 26% in 'Pink Panther' and 16% in 'Red hawk' (Table 2).

The interactions between growth stages and market class, and growth stages and cultivars within market class were also found statistically significant, but the effect of growth stages on market class or cultivars within market classes were same (Table 2). If there was a significant difference, the RUN values were found higher at R_2 stage.

The dry bean plants could get only a little amount of N from the growing media and seed, and for the rest amount of N, N_2 fixation was the only source. As a result, the plants produced

higher amounts of ureides compared to the amount of NO_3^--N available from the growing media, which yielded higher RUN values. The RUN values for different cultivars in this study were in the range similar to the RUN values reported (80% to 100%) in an earlier study on soybean cultivars conducted by Herridge and Peoples (1990) under a similar growing condition with little or no N.

Percent N derived from atmosphere (%Ndfa)

Statistically significant differences were found between mean %Ndfa values at two growth stages and among the dry bean market classes (Table 3). Navy (90.4) had the highest %Ndfa value at both the growth stages followed by pinto (80.6), kidney (59.6), and black (48.8) at V_3 stage; and pinto (92.3), black (75.5), and kidney (70.6) at R_2 stage. The %Ndfa values significantly improved at R_2 stage from the V_3 stage (Table 3.3); highest increment was found in the black (55 percent) market class followed by kidney (19 percent), pinto (15 percent), and navy (8 percent). Significant differences were also found for growth stage and market class interactions, but the ranking of market classes for %Ndfa at both the stages were found same, and higher values were found at R_2 stage.

Navy and pinto cultivars were found to have higher %Ndfa values than earlier reported values (between 9 to 79 percent), but the black and kidney cultivars had %Ndfa values in the range reported in earlier studies (Kipe-Nolt et al., 1993; Müller and Pereira, 1995; Farid and Navabi, 2015). The main reason was the source of N. The dry bean plants got almost its entire assimilated N through N_2 fixation, which resulted in higher isotopic dilution. Rennie (1982) also reported that using non-nodulating mutant of plant under study (here, R99, a white navy mutant line) tended to overestimate the N_2 fixation.

Table 3.2: Variations in mean plant dry matter yield and relative ureide N (%) of twelve dry bean cultivars under Pinto, Navy, Kidney and Black bean market classes at two growth stages

Market Class	Cultivar	Plant dry matter (g plant^{-1})		Relative Ureide N (%)	
		V_3 stage	R_2 stage	V_3 stage	R_2 stage
Pinto	La Paz	2.36b (0.14)	2.85 (0.18)	63.8b (3.3)	70.2b (1.7)
	Lariat	2.33b (0.20)	3.18† (0.47)	88.8a (2.9)	92.3a (3.3)
	Windbreaker	2.86a (0.31)	3.01 (0.25)	88.3a (1.9)	90.5a (5.1)
	Mean	2.52A (0.34)	3.01A‡ (0.35)	80.3A (11)	84.4 (11)
Navy	Ensign	2.48 (0.09)	2.60 (0.36)	80.8 (3.7)	88.1 (3.2)
	Medalist	2.39 (0.07)	2.23 (0.15)	78.8 (4.7)	87.8 (3.4)
	T9905	2.49 (0.19)	2.61 (0.24)	80.3 (4.3)	86.9 (3.1)
	Mean	2.45B (0.13)	2.48B (0.31)	80.0A (4.3)	87.6‡ (3.2)
Kidney	Foxfire	3.05 (0.08)	2.91 (0.07)	60.0b (5.8)	85.1† (1.1)
	Pink Panther	3.13 (0.13)	3.05 (0.21)	68.8a (3.7)	86.6† (1.7)
	Red hawk	2.94 (0.13)	2.88 (0.17)	69.2a (5.9)	80.2† (1.9)
	Mean	3.04A (0.12)	2.94A (0.18)	66.0B (6.8)	84.0‡ (3.2)
Black	Eclipse	2.68 (0.08)	2.63 (0.16)	78.9 (2.6)	87.0 (1.1)
	Loreto	2.60 (0.06)	2.78 (0.11)	78.2 (3.4)	87.0 (1.3)
	Zorro	2.77 (0.14)	2.93 (0.12)	83.7 (3.7)	89.7(1.0)
	Mean	2.68A (0.14)	2.73A (0.31)	94.2A (3.2)	96.7‡ (0.5)
Growth stage			**		**
Market class			**		**
Cultivar (Market class)			**		**
Growth stage * Market class			**		**
Growth stage*Cultivar (Market Class)			*		**

Means followed by same lowercase letter(s) are not statistically significant among the cultivars within a market class at $P \leq 0.05$ under a specific growth stage; Means followed by same uppercase letter are not statistically significant among market classes at $P \leq 0.05$ under a specific growth stage; Means with † are statistically dissimilar at $P \leq 0.05$ among the cultivars within the market classes between two growth stages; Means with ǂ are statistically dissimilar at $P \leq 0.05$ among the market classes between two growth stages; Standard deviations are given in the parenthesis; * Treatment main effect statistically significant at $P \leq 0.05$; ** treatment main effect statistically significant at $P \leq 0.01$; NS: treatment main effect statistically non-significant;

Table 3.3: Variations in mean percent N derived from atmosphere (%Ndfa) and N_2 fixed (mg N plant^{-1}) of twelve dry bean cultivars under Pinto, Navy, Kidney and Black bean market classes at two growth stages

Market Class	%Ndfa		N_2 fixed (mg N plant^{-1})
	V_3 stage	R_2 stage	
Pinto	80.6b (9.7)	92.3a† (4.8)	68.0a (26)
Navy	90.4a (8.5)	97.4a† (2.8)	58.8a (25)
Kidney	59.6c (6.0)	70.6b† (2.9)	33.4b (6.2)
Black	48.8d (7.5)	75.5b† (3.5)	34.7b (13)
Growth stages	**		NS
Market Class	**		**
Growth Stage × Market Class	**		NS

Means followed by same lowercase letter(s) are not statistically significant among the cultivars within a market class at $P \leq 0.05$ under a specific growth stage; Means with † are statistically dissimilar at $P \leq 0.05$ among the market classes between two growth stages; Standard deviations are given in the parenthesis

* Treatment main effect statistically significant at $P \leq 0.05$; ** treatment main effect statistically significant at $P \leq 0.01$; NS: treatment main effect statistically non-significant;

Variations in the %Ndfa can also be attributed to the differences in the symbiotic efficiencies between the dry bean plant of a specific market class and the specific rhizobia strain(s) in the added through commercial liquid inoculant. Better symbiotic compatibility results in higher %Ndfa values, and it was reported earlier that cultivars exhibit differences in their compatibility with a rhizobia strain (Weiser et al., 1985).

N_2 fixation

Nitrogen fixed by different dry bean market classes varied significantly (Table 3.3). The black and kidney bean classes fixed significantly lower amounts of N_2 than pinto and navy classes. Pinto (68.0 mg N plant^{-1}) market class fixed highest amount of N_2, followed by navy (58.8 mg N plant^{-1}), black (34.7 mg N plant^{-1}), and kidney (33.4 mg N plant^{-1}) market class (Table 3.3). These values of N_2 fixed were in the range of earlier reported values for dry bean

cultivars. Rennie and Kemp (1982) reported values in the range between 17 mg N plant^{-1} to 166 mg N plant^{-1} for N_2 fixed, while Akter et al. (2014) reported values between 4 mg N plant^{-1} and 129 mg N plant^{-1}.

The dry bean cultivars exhibit differences in the growth habit, duration of the growth stages, plant architecture, seed size and more importantly, their requirement for N (Table 3.1). Cultivars with indeterminate growth habit were found to fix more atmospheric N_2 than determinate cultivars due to longer period of N_2 fixation in earlier studies (Piha and Munns, 1987; Farid and Navabi, 2015). In our study, we found that kidney bean cultivars with determinate growth habit had the lowest mean amount of N_2 fixed, and other market classes with indeterminate growth habit fixed higher amount of N_2 (Table 3.3).

Limited supply of mineral N from the growth medium forced the plants to fix more N_2 from atmosphere, but the plants were unable to fix enough N_2 to support optimum plant growth in the later growth stages. The duration of growth stages was shortened; plants switched to reproductive stage earlier than normal growing conditions, and showed symptoms of senescence early. Similar observations were also reported earlier (Ohyama, 2010). Moreover, poor plant growth and functioning resulted in limited supply of soluble carbohydrates (malate, succinate etc.) to the root nodule impeded the normal functioning of the bacteria inside the bacteroid tissue, and ultimately disrupted the N_2 fixation (Mengel et al., 2001).

Correlation

Significant correlation ($P \leq 0.01$) was found in between the %Ndfa and N_2 fixed ($r = 0.65$) (Table 3.4). The plants that could derive higher amount of atmospheric N_2 had better vegetative growth, and fixed higher amount of N_2. The RUN values were significantly ($P \leq 0.01$) correlated with %Ndfa, but the correlation was found weak ($r = 0.376$). As reported in a previous

study, remobilized N from older leaves increases the ureide concentration in the shoots and leaves during the early pod filling in N_2 fixing plants (Díaz-Leal et al., 2012). Therefore, the ureide concentration and %Ndfa data from stable isotope dilution study did not correlate well at the later growth stages, and gave low correlation values.

Table 3.4: Pearson correlation coefficients (r) between plant dry matter, relative ureide N, percent N derived from atmosphere (%Ndfa) and N_2 fixed by dry bean cultivars

	Plant dry matter	Relative Ureide N	%Ndfa	N_2 fixed
Plant dry matter	-	-0.033^{NS}	-0.228*	0.079^{NS}
Relative Ureide N	-0.033^{NS}	-	0.376**	0.311**
%Ndfa	-0.228*	0.376**	-	0.645**
N_2 fixed	0.079^{NS}	0.311**	0.645**	-

* Correlation statistically significant at $p \leq 0.05$; ** statistically significant at $p \leq 0.01$; NS: Non-significant;

Conclusion

The new liquid *Rhizobium* inoculant helped in nodulation in dry bean roots and can be used as an alternative of existing peat-inoculant. This inoculant should further be thoroughly tested against other available inoculant formulations, and under different environmental conditions.

Severe N stress during early growth stages found to be irrecoverable for all the dry bean cultivars within market classes even through later addition of mineral N. The market classes varied in their potential for SNF. Among the dry bean market classes, the pinto and navy bean classes fixed significantly higher amount of N_2 compared to black and kidney market classes. Therefore, to get higher amount of N_2 fixed, pinto and navy bean cultivars should be opted, for both breeding and cultivation.

The RUN values were not well correlated with %Ndfa or N_2 fixed, and did not show significant variation among market classes at R_2 stage, when there was significant variation for

SNF among dry bean market classes. Therefore, the ureide method could not assess the SNF by dry bean cultivars as it did for soybean. This method should be calibrated for dry beans in order to consider it as a reliable tool to quantify N_2 fixation ability of dry bean cultivars. The %Ndfa calculated from isotope dilution was significantly correlated with the N_2 fixed by different dry bean market classes. Isotope dilution method using ^{15}N is the most preferable technique to estimate the N_2 fixation by dry bean.

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CHAPTER IV: SYMBIOTIC NITROGEN FIXATION IN DRY BEAN (*PHASEOLUS VULGARIS* L.) CULTIVARS INOCULATED WITH *RHIZOBIA* IN THE NORTHERN GREAT PLAINS

Abstract

Symbiotic Nitrogen Fixation (SNF) is an environmentally safe source of N to the crop plants and is carried out by rhizobia in symbiosis with a specific legume such as dry beans (*Phaseolus vulgaris* L.). SNF potentials of eight dry bean cultivars from pinto and kidney market classes were studied in a field experiment using a split plot randomized complete block design at Prosper, ND in 2016 and Felton, MN in 2017. Dry bean seeds were inoculated with liquid and peat-based *Rhizobia* inoculants prior to sowing in the field. Plant tissue samples were collected at the third trifoliate stage (V_3) and late flowering stage (R_2) to determine percent N derived from atmosphere (%Ndfa) using the stable isotope dilution technique. Control (non-inoculated) treatment (1.63 Mg ha^{-1}) had significantly higher seed yield than the inoculation treatments (1.38 Mg ha^{-1}) across the years. Overall, after combined analysis, peat inoculation resulted into 14% higher %Ndfa at V_3 stage than liquid inoculation. Between market classes, pinto had 12% higher %Ndfa than kidney, and probably translated into higher seed yield in pinto. Significant differences in seed yield was found among kidney cultivars [Montcalm (1.43 Mg ha^{-1}) > Pink Panther (1.18 Mg ha^{-1}) > Foxfire (0.91 Mg ha^{-1}) > Redhawk (0.80 Mg ha^{-1})] irrespective of inoculation treatments, but not among pinto cultivars. Also, kidney had significant interaction with inoculation treatments and had 4% higher seed yield under liquid inoculation compared to peat. Our results suggest that pinto cultivars had higher N_2 fixing potential (%Ndfa) compared to kidney and performance of rhizobia inoculant is market class specific.

Keywords: Symbiotic Nitrogen Fixation; Rhizobia; Dry bean; Inoculation; Isotope dilution; %Ndfa

Introduction

Nitrogen (N) is a structural constituent protein, one of the most important structural components of any living organism. Symbiotic nitrogen fixation (SNF) is the prime source of environment-friendly N to the agronomic crops. SNF also improves soil fertility, maintains N-balance in soil, and regulates N availability to the crops (Triboi and Triboi-Blondel, 2014; Ronner et al., 2016). Legumes take part in symbiosis with rhizobia (*Rhizobia*, *Bradyrhizobia*, *Sinorhizobia*) and are reported to fix about 20×10^6 Mg of N in soils per year into the world agricultural systems (Herridge et al., 2008). Thus, in order to increase SNF, inoculation of legume seeds with rhizobia can be beneficial. Legume species and genotypes respond variably to rhizobia inoculation, depending on their symbiotic compatibility with rhizobial strain in symbiosis and environmental conditions (Heilig et al., 2017; Youseif et al., 2017).

Dry bean (*Phaseolus vulgaris* L.) is the most important food-legume crop and the largest source of plant proteins along with low-fat carbohydrates, fiber, folates, minerals, thiamine and riboflavins; that contributes directly to global food-sufficiency (Farid et al., 2016). Dry beans have been generally reported as 'poor N_2 fixers' (30-50 kg N ha^{-1}) compared to other grain legumes such as soybean (*Glycine max* L. Merr.) and lentil (*Lens culinaris* Medik.), which fix around 80-100 kg N ha^{-1}; still, dry beans add about 6×10^5 Mg N annually in the agricultural systems globally (Herridge et al., 2008; Farid and Navabi, 2015). Earlier studies reported that when dry bean seeds were inoculated with rhizobia, dry bean cultivars fixed higher amounts of N_2 (as high as 124 kg N ha^{-1}) (Rennie and Kemp, 1984); however, the inoculation effect is not consistent globally. Poor nodulation and non-significant response of dry bean under rhizobia

inoculation for SNF and seed yield in the field trials are well-reported globally (Buttery et al., 1987; Buetow et al., 2017; Denton et al., 2017).

Along with environmental factors like extreme pH (both acidic and alkaline conditions), water stress and salinity, native rhizobia population in soil influences the response of rhizobia inoculation (Singleton and Tavares, 1986; Farid et al., 2017). However, in dry bean growing areas of North Dakota (ND) and Minnesota (MN), environmental stresses such as heavy rainfall with subsequent flooding, heavy wind, and biotic stresses such as weeds and diseases such as white mold (*Sclerotinia sclerotiorum*), common bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli*), anthracnose (*Colletotrichum lindemuthianum*), root rot (*Fusarium solani* f. sp. *phaseoli* or *Rhizoctonia solani*) severity, are the potential factors that limit nodulation and N_2 fixation as well (*Knodel et al.*, 2017). Ultimately, the positive inoculation responses depend on the genotype, genotype × rhizobia strain interaction, inoculant formulation, environmental conditions, and the application method (Thilakarathna and Raizada, 2017).

Among all the dry bean producing countries, United States (US) ranks fourth and produced 1.3×10^5 Mt of dry beans in 672, 590 ha of area in 2016 (*FAOSTAT*, 2014; *USDA ERS* 2016). In the US, ND is the largest producer (38% of the national production) of dry beans followed by Michigan (13%), MN (10%), and Nebraska (9%) (NASS, 2016). Unfortunately, only 14% dry bean growers in ND and 16% growers in MN used rhizobia inoculants in their field (Knodel et al., 2017). In addition, the available peat-based rhizobia inoculants for dry beans clog the pores of precision air-seeder planters commonly used in these dry bean growing areas, which increases planting time and labor (for seed inoculation), thus discourages dry bean growers to adopt inoculation (Singleton et al., 2002). In recent years, liquid rhizobia inoculation has resulted in seed yields either comparable with or higher than peat-based inoculation in some

grain legumes such as soybean, field pea (*Pisum sativum* L.) and lentil (Ciafardini and Barbieri, 1984; Hynes et al., 1995), but no liquid rhizobia inoculant is commercially available for dry bean in the Northern Great Plains.

The use of ^{15}N, a stable isotope of N, is the most reliable and most accurate approach to quantify N_2 fixation and it has been extensively reviewed (Chalk, 1985; Witty et al., 1988). Among several ^{15}N-isotopic methods, the 'Isotope dilution technique' has high efficiency in discriminating the N_2 fixed from the atmosphere and the N taken up from the soil as reported several published literatures (Danso, 1986; Barrie, 1991). A non-fixing reference plant is needed to quantify %Ndfa and both the fixing and reference plants should uptake N from the soil with same ^{15}N isotopic composition (Boddey et al., 1984).

As genotypic variation exists for SNF in dry beans, this study was designed to estimate the N_2 fixation ability of different cultivars. Our objectives were i) to determine %Ndfa of different dry bean cultivars at two different growth stages, V_3 and R_2 stage, ii) quantify SNF for different dry bean cultivars, and iii) compare different rhizobia inoculants based on SNF and seed yields.

Materials and methods

Study site, design, and environment

A two-year field study was conducted during the growing seasons of 2016 and 2017 to check the potential of the *Rhizobia* inoculant and SNF of dry bean cultivars. In 2016, the experiment was carried out at the NDSU Research Experimental Station at Prosper, ND (47.02, -97.15). In 2017, the experiment was in a farmer's field with no dry bean history at Felton, MN (47.14, -96.57). The initial soil parameters for both the fields are reported in the Table 1. The monthly mean air temperatures during the crop-growing season (from May to October) were

15.7˚C in 2016 and 8.9˚C in 2017; the amounts of total precipitation received during the growing season were 366 mm in 2016 and 335 mm in 2017 (Table 2). The experimental plots were laid out using a split-plot design where inoculant treatments were considered as main-plot treatment and dry bean cultivars as sub-plot treatments with four replications. Individual plot dimensions were 4*3 m^2 with four rows in 2016 and 7.6*3.4 m^2 with six rows in 2017.

Treatments

Three rhizobia inoculation treatments including a non-inoculated 'control', a commercial peat (N-Dure™, Verdesian™) and a commercial liquid (Excalibre SA™, ABM™) inoculants were used as pre-sowing seed inoculation in both years. The seeds were treated with rhizobia inoculant rate as per instructions in the manufacturers' protocol. Seeds with a single inoculation treatment were sown at a time and the planter was washed with 95% ethanol after application each inoculation treatment to avoid contamination. Seeds were planted at a depth between 2.5-3.8 cm.

Eight cultivars, four from pinto and kidney market classes (Table 2) were selected based on their popularity among dry bean growers in North Dakota and Minnesota area (most areas planted under the specific cultivar) as reported in 2014 Dry bean growers' survey (Knodel et al., 2015). No additional fertilizers were applied to the soil. Herbicides (Basagran® and Raptor®) were applied once and hand weeding at regular interval was done to control weeds until harvesting.

Initial rhizobia population

Soil samples were collected from the experimental fields before sowing of dry bean seeds each year using a clean soil probe that has been washed with soap and water, and then dried in air. Ten soil cores were taken randomly from the experimental plots at a 6-inch depth, broken up

with hands and then screened through a 2 mm sieve. From this sample, subsamples were taken for the rhizobia count. Background rhizobia populations in both the fields were estimated using these sub samples using most probable number (MPN) method (Brockwell, 1963). Rhizobia population in 2016 was estimated to be 19 rhizobia per g of soil, but in 2017, the rhizobia population could not be estimated and considered as zero.

Table 4.1: Location and initial soil parameters of the experimental fields in 2016 and 2017

	2016	**2017**
Location (Latitude, Longitude)	Prosper, ND (47.02, -97.15)	Felton, MN (47.14, -96.57)
Soil Series	Glyndon-Tiffany silt loam	Colvin silty clay loam
Previous crop	Wheat	Corn
pH	6.61	7.87
Electrical Conductivity (dS m^{-1})	1.03	0.98
Bulk density (g cm^{-3})	1.34	1.21
Organic Carbon (%)	4.00	4.77
Sand (%)	11.4	3.70
Silt (%)	51.9	55.0
Clay (%)	36.7	41.3
Nitrate-N (Kg ha^{-1}) (top 30 cm)	16.8	52.3
Olsen-P (ppm)	37.0	42.8
Available K (ppm)	312	441
Background ^{15}N (atom%)	0.37	0.37
Planting Date	18th May	25th May
Harvesting Date	7th September	15th September

Stable isotope enrichment and reference plant

Dry bean plants were supplied with $K^{15}NO_3$ (enriched with 99.2 atom % ^{15}N) for 5 atom% ^{15}N enrichment and the solution was applied uniformly to the soil near the plants at 21 days after planting (DAP) for subsequent stable isotopic analysis following the isotope dilution technique (McAuliffe et al., 1958; Chalk, 1985). A non-nodulating navy dry bean mutant cultivar 'R99' (Shirtliffe and Vessey, 1996) was selected as the 'non-fixing' reference plant and maintained along with the dry bean cultivars in the field.

Table 4.2: Description of cultivars within pinto and kidney market classes of dry bean (*Phaseolus vulgaris* L.) selected in the study

Market Class	Cultivars	Origin	Dry bean plant type	PVP #
Kidney	Redhawk	Mich. AES	B (I)	200000264
	Pink Panther	Seminis	B (I)	200300266
	Foxfire	ADM/Seedwest	B (I)	9200069
	Montcalm	Mich. AES	B (I)	*PVP 94 Protected*
Pinto	La Paz	Provita	USV (II)	200500219
	Lariat	NDSU	USV (II)	200800305
	Windbreaker	Seminis	UV (IIb)	200500105
	ND-307	NDSU	UV (IIb)	200900009

UV: Upright Vine, USV: Upright short Vine, B: Bush; I: Bush- Determinate, II: Upright – Indeterminate; IIb: Upright indeterminate, but prostrated/ decumbent in some environments; III: prostrated/ Decumbent – Indeterminate; PVP #: Plant Variety Protection Number; Source: Drybean Production Guide, Edited by H. Kandel, NDSU Extension Service (2013)

Plant sampling and data collection

Plant samples were collected twice, one at third trifoliate (V_3) stage and another one at late flowering stage (R_2). These two growth stages for sampling were selected as N_2 fixing activity has been reported to be higher in these two stages of growth (Franco et al., 1979). Both non-fixing and N_2 fixing legume plant samples were collected on the same day. Plant shoots

were collected and root nodules were counted at both the growth stages. Fresh plant-shoot samples were weighed, and then dried at 55-60˚C for 4 days.

Isotope dilution technique

Dried plant shoot tissues were ground to pass 0.02 mm screen, and about 3-4 mg of ground samples were encapsulated into tin (Sn) capsules. Encapsulated samples were packed in 96-well trays, and sent to UC Davis Stable Isotope facility. Samples were analyzed for isotopic ratio (i.e. ^{15}N: ^{14}N) using a continuous flow Isotope Ratio Mass Spectrometer (PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 IRMS). Percent N derived from atmosphere (%Ndfa) was used as an indicator of SNF and was calculated from 'atom% ^{15}N' values of plant samples using the following formula

$$\%\, Ndfa\ = \left(1 - \frac{atom\%\ 15_N\ excess\ in\ N_2 - fixing\ plant}{atom\%\ 15_N\ excess\ in\ reference\ plant}\right) \times 100$$

The 'atom % excess ^{15}N' is the ^{15}N enrichment above the background. i.e. atmosphere (the value is 0.3663%). The %Ndfa was calculated from ^{15}N atom% excess of plant shoot tissue of the dry bean cultivars. N_2 fixation was calculated using the following formula:

$$N_2\ fixed\ (kg\ ha^{-1}) = Dry\ matter\ yield\ (kg\ ha^{-1}) * \%Ndfa * N\ in\ plant\ tissue\ (\%)$$

Statistical analysis

The data of %Ndfa, N_2 fixed, and seed yield at the two sampling stages across two years were analyzed for analysis of variance (ANOVA) using generalized linear model using 'proc glm' procedure in SAS 9.4 software (SAS Institute, 2013). Environments (study year and location combined effect), inoculation treatments, market classes, cultivars nested in market class, were fixed factors, and the replications in each study site along with the interaction effects (growth stages × market class and growth stages × cultivars nested in market class) were considered as random factors. Combined analyses were also done pooling the data across two

years together as the mean square error values for each attribute were in the permissible range for combined analyses. Generalized Linear Model was used to analyze the pooled data using 'proc glm' procedure. Least square means were computed using 'lsmeans' statement, standard errors of least squared means were estimated, and pairwise significant difference tests were performed using the 'adjust=TUKEY' statement at 95% significance level in SAS 9.4 software (SAS Institute, 2013).

Results and discussion

Significant differences were found among the different inoculation treatments and market classes for %Ndfa at V_3 stage, but at R_2, only market classes were significantly different across both environments (Table 3). Among the inoculation treatments, liquid (56%) had significantly lower %Ndfa at V_3 stage than peat (65%) and control (66%), and no significant difference was found between the latter two treatments (Table 4).

In this study, rhizobia inoculation did not significantly increase %Ndfa compared to control. Similar observations were reported in a multi-site trial where significant increase in %Ndfa was found in some of the sites, but not in the other sites (Denton et al., 2017). Another study reported that the N derived from N_2 fixation was not significantly different for all the inoculation treatments including control across all study conditions even when there were differences in the number of native rhizobia and the soil available N levels (Peoples et al., 1992).

Table 4.3: Probability levels for significant factors for percent nitrogen derived from atmosphere (%Ndfa) at third trifoliate (V_3) and late flowering (R_2) stage, N_2 fixed (kg ha^{-1}), and seed yield (kg ha^{-1}) after combined analyses

	Ndfa at V_3 (%)	Ndfa at R_2 (%)	Dry matter (kg ha^{-1})	Seed yield (kg ha^{-1})
Environment	<.0001**	<.0001**	<0.0001**	<.0001**
Inoculation	<.0001**	0.2589 ^{NS}	0.0259*	0.0009**
Market Class	<.0001**	0.0121*	0.0065**	<.0001**
Cultivar (MC)	<.0001**	0.3254 ^{NS}	0.0924^{NS}	<.0001**
Inoculation*Market Class	0.0041**	0.2362 ^{NS}	0.3777^{NS}	0.0045**
Inoculation*Cultivar (MC)	0.0465*	0.4687 ^{NS}	0.7620^{NS}	0.9685 ^{NS}
Environment *Inoculation	0.7190^{NS}	0.0564 ^{NS}	0.3848^{NS}	0.0930 ^{NS}
Environment *Market class	0.0029**	0.9448 ^{NS}	0.0811^{NS}	0.3504 ^{NS}
Environment *Cultivar (MC)	0.0001**	0.0864 ^{NS}	0.3603^{NS}	0.0170*
Environment *Inoculation*Market Class	0.2395 ^{NS}	0.0025**	0.2824^{NS}	0.0258*
Environment *Inoculation*Cultivar (MC)	0.0291*	0.4092 ^{NS}	0.9825^{NS}	0.1686 ^{NS}

* Treatment main effect statistically significant at $P < 0.05$; ** treatment main effect statistically significant at $P < 0.01$; NS: treatment main effect statistically non-significant

Table 4.4: Variation in mean percent N derived from atmosphere (%Ndfa) at V_3 stage and seed yield (kg ha^{-1}) among inoculation treatments after combined analysis

Inoculation	%Ndfa (V_3)	Seed Yield (kg ha^{-1})
Control	65.7a (4.1)	1632a (69.5)
Liquid	55.7b (3.8)	1336b (86.1)
Peat	65.0a (3.5)	1411b (105)

Standard errors are given in parenthesis below mean values for the significant effects; Treatment means followed by same lowercase letter(s) are not significantly different at $P < 0.05$

Table 4.5: Variation in mean percent N derived from atmosphere (%Ndfa) at V_3 and R_2 stage and seed yield (kg ha^{-1}) between market classes after combined analysis

Market Class	%Ndfa (V_3)	%Ndfa (R_2)	Seed yield (Mg ha^{-1})
Kidney	55.6b (2.4)	73.5b (1.5)	1.08b (0.06)
Pinto	67.8a (1.9)	78.0a (1.4)	1.84a (0.05)

Standard errors are given in parenthesis below mean values for the significant effects; Treatment means followed by same lowercase letter(s) are not significantly different at $P < 0.05$

Table 4.6: Variation in mean seed yield (kg ha^{-1}) among kidney cultivars after combined analysis

Cultivar	Seed Yield (Mg ha^{-1})
Foxfire	0.91b (0.12)
Montcalm	1.43a (0.12)
Pink Panther	1.18ab (0.15)
Redhawk	0.80b (0.08)

Standard errors are given in parenthesis below mean values for the significant effects; Treatment means followed by same lowercase letter(s) are not significantly different at $P < 0.05$

There might be several reasons behind this outcome as reported in earlier studies. The native rhizobia that reside in soil are also capable of producing nodules in dry bean roots and take part in the SNF. There are several studies reported earlier that mentioned that native rhizobia are capable of fixing substantial amount of N_2 from atmosphere (Martins et al., 2003; Koskey et al., 2017). In our study, we found 19 rhizobia g^{-1} of soil in 2016, which indicates there was a high chance that in control plots, dry bean plants would be nodulated by the native rhizobia strains, and the native strains would have helped in SNF. Although, we could not estimate the initial rhizobia population in 2017, but nodulation was found in the control plots too. In addition, rhizobia strains that are chosen for commercial inoculants not only based their symbiotic efficiency, instead, mostly based on their high reproducing ability, high growth rate or surviving ability in variable conditions (Bergersen et al., 1971). There are several other factors

such as competitive ability with other strains, speed of nodule production, ability to fix N_2 under wide range of soil thermal and moisture conditions, resistance to chemicals and acidic conditions in soils, which may not correlate well with N_2 fixation or seed yield (Date, 2000). Thus, in the inoculated plots, no significant increase in %Ndfa was found.

Table 4.7: Variation in mean seed yield (kg ha^{-1}) among inoculation and market class interactions after combined analysis

Market Class	Inoculation		
	Control	Liquid	Peat
Kidney	1.40bA (0.07)	0.94bB (0.07)	0.91bC (0.08)
Pinto	1.87a (0.05)	1.74a (0.06)	1.92a (0.08)

Standard errors are given in parenthesis below mean values for the significant effects; Treatment means followed by same lowercase letter(s) are not significantly different between cultivars and treatment means followed by same uppercase letter(s) are not significantly different among inoculation treatments at $P < 0.05$

N_2 fixation activity depends on the symbiotic efficiency between the rhizobium strain and the legume genotype (Thilakarathna and Raizada, 2017). Higher %Ndfa at V_3 stage was obtained with peat inoculation compared to liquid inoculation which possibly indicated better symbiotic efficiency of rhizobia strains of peat inoculant with the dry bean genotypes compared to liquid inoculant strains (Table 4). It is long known that different *R. phaseoli* strains produced significantly different number and amount of nodules in different dry bean genotypes (Weiser et al., 1985). Also, there is always a possibility that the liquid inoculant probably did not adhere to the dry bean seed coat properly during application through the seeder and carried lesser number of rhizobia per seed than the peat inoculants as less number of rhizobia adhering to the seeds may limit nodulation (Date, 2000; Deaker et al., 2004). In addition, the carrier material influences the longevity of the bacteria, thus influence numbers of viable rhizobia available

during nodulation (Deaker et al., 2004); these two different formulations, peat and liquid, might have introduced differences in their supply of viable rhizobia for symbiosis.

In our experiment, control had higher %Ndfa than liquid inoculation and comparable %Ndfa with peat inoculant, which possibly indicated that even native rhizobia were probably more efficient dry bean symbionts than the liquid inoculant strains, which resulted in higher %Ndfa. In the control plots, probably only native rhizobia nodulated dry bean plants and took part in symbiosis as no commercial inoculant was applied , and it was also reported that there are six different genera of rhizobia that can nodulate dry bean (vanBerkum et al., 1996; Herrera-Cervera et al., 1999). Previous studies have also shown that the native rhizobia strains performed better than the inoculant strains (Tajini et al., 2008; Mulas et al., 2015). The influence of native rhizobia population on effectiveness of rhizobia inoculation was reported earlier and it was concluded that N requirements were varied based on native rhizobia population and their effectiveness as it influences symbiotic efficiency and subsequent N_2 fixation (Argaw and Tsigie, 2015), but in this study, the rhizobia species in the nodules of dry bean plants were not determined.

In the liquid inoculated plots, the native and inoculant rhizobia co-existed as it was assumed that native rhizobia population was not restricted by any means such as chemicals and agronomic practices, and competed for symbiosis with the dry beans, for nutrition and habitat (Mengel et al., 2001). As a large population of rhizobia was added as liquid inoculation in the proximity of the seeds during sowing, probably dry bean plants in the liquid inoculated plots were dominantly nodulated by liquid inoculant *Rhizobium* strains. The speed of nodulation, i.e. how fast the rhizobia can nodulate the plant roots, is a major factor that influences competitive ability of rhizobia (de Oliveira and Graham, 1990) and there was higher probability for the liquid

inoculant rhizobia strain to infect and nodulate the plant roots before the native rhizobia strains. Lower %Ndfa and subsequent lower N_2 fixation in these plots might indicate the lower symbiotic efficiency of the liquid inoculant strain with the dry bean cultivars used in these study (Table 4; A8, A11).

Between the two market classes under study, pinto (68 at V_3 and 78 at R_2 stage) had significantly higher %Ndfa at V_3 and R_2 stage compared to kidney (56 at V_3 and 74 at R_2 stage) (Table 5). Differences in %Ndfa between kidney and pinto market classes and among commercial cultivars within each market class (Table A6, A7) can be attributed to their genetic makeup and symbiotic efficiencies with rhizobia strains as reported in earlier studies on dry beans (Rennie and Kemp, 1982; Weiser et al., 1985). The market classes and the cultivars also differ in their growth habits and agronomic performance in different growing conditions, thus acquisition of N from the environment i.e. soil and atmosphere was also different (Table 2) (Hardarson et al., 1993). The market classes and cultivars had differences in their biomass and seed yield (Appendix A9, A10), therefore, their N requirement and N_2 fixation activities were different as concluded in an earlier study (Denton et al., 2017).

Seed yields among inoculation treatments, between the market classes and among the kidney cultivars were significantly different across environments (Table 3). Among the inoculation treatments, peat and liquid had significantly lower seed yield compared to control (Table 4). Rhizobia inoculation did not increase seed yield significantly in our experiments in both years as reported in earlier studies, and specially studies that took place in the northern great plains (Bengtsson, 1989; Vessey, 2003; Denton et al., 2017). Contrastingly, in most of the rhizobia inoculation studies, significantly higher seed yields were obtained with inoculation (Hungria et al., 2003; Zhang et al., 2010). It has been also reported that rhizobia inoculation was

mostly effective to increase in seed yield when applied to a field for the first time (Bengtsson, 1989; Vessey, 2003). Similarly, our field experiment in 2017 were laid out in a field without any dry bean history, but no significant increment in %Ndfa or seed yield was found. Another study reported that rhizobia inoculation increased grain yield of pigeon pea (*Cajanus cajan*) when the N rate was lower (15 kg ha^{-1}), but did not increase yield significantly when the N rate was doubled (Singh et al., 1998). Likewise, in our study, higher initial soil NO_3^--N availability was found in 2017 compared to 2016 (Table 1), and lower mean %Ndfa values were found in 2017 than 2016 (Supplementary table 2).

Between the market classes, pinto (1.84 Mg ha^{-1}) had significantly higher yield than kidney (1.08 Mg ha^{-1}) (Table 5). In the dry bean varietal trials organized by NDSU, similar reports of pinto yielding more seeds than kidney were published, but seed yield values reported were higher than the values found in our field trials (Kandel et al., 2016). Cultivars within kidney market class showed significant variation; Montcalm (1.43 Mg ha^{-1}) had highest seed yield followed by Pink Panther (1.18 Mg ha^{-1}), Foxfire (0.91 Mg ha^{-1}), and Redhawk (0.80 Mg ha^{-1}) (Table 6). Seed yield differences among market classes and cultivars could be due to genotypic differences as reported in previous studies, but symbiotic efficiency and acquiring N from atmosphere can be another possibility that influenced seed yield (Hardarson et al., 1993). Similarly, in our study, higher %Ndfa at both the growth stages were found in pinto compared to kidney which might have translated into higher seed yield in pinto compared to kidney (Table 5).

Interaction between inoculation and market class for seed yield was found significant and true (not due to magnitude) (Table 3). Under all the inoculation treatments, pinto had higher seed yield than kidney. On the other hand, kidney had highest seed yield under control (1.4 Mg ha^{-1}), followed by liquid (0.94 Mg ha^{-1}) and peat (0.91 Mg ha^{-1}) inoculation (Table 7). For some

kidney cultivars, a negative interaction was found between inoculant and seed germination in some cultivars, which eventually resulted in poor plant stands and dry matter yield for Pink Panther and Redhawk under inoculation (Supplementary Table 9); poor plant stand due to poor germination was another plausible reason for poor seed yield in specific kidney cultivars.

Most of the previous studies that reported increment in SNF potential of dry bean cultivars with rhizobia inoculation did not consider the effect of market classes, and compared the cultivars irrespective of their market classes (Rennie and Kemp, 1982; Hardarson et al., 1993). However, the phenotypic (growth habit, N uptake and accumulation pattern, root growth, and agronomic behavior in different environmental conditions) and genotypic variations among dry bean market classes are too variable to be considered as similar study system, however in this study, we compared market classes of dry beans and the cultivars within each market classes (Table 2). Future studies should consider different dry bean market classes with variable growth habits and more growth stages to understand the need of rhizobia inoculation and reveal the detailed physiology behind SNF in dry beans.

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CHAPTER V: EXPRESSION DYNAMICS OF RHIZOBIAL NIFH AND DRY BEAN (*PHASEOLUS VULGARIS* L.) NR AND GS GENES AT DIFFERENT GROWTH STAGES IS GENOTYPE DEPENDENT AND CORRELATE WITH AMOUNT OF TOTAL NITROGEN FIXED

Abstract

Selection of dry bean (*Phaseolus vulgaris* L.) cultivars that induce higher levels of symbiotic nitrogen fixation (SNF), an environment-friendly source of nitrogen (N), in association with *Rhizobium phaseoli* could reduce the application rate of N fertilizer. The bacterial *nifH* gene is a 'marker' for SNF, which provides organic N that can be assimilated into plant proteins by the N-assimilatory enzymes nitrate reductase (encoded by the *NR* gene) and glutamine synthetase (encoded by the *GS* gene). However, research showing that host-symbiont genotype specific interactions determine *nifH* gene expression levels that correlate with phenotypic expression (i.e. the amount of N_2 fixed by the plant) in legume crops including dry bean is lacking. To fill this knowledge gap, bacterial *nifH* gene expression levels were correlated with the expression of the N-assimilatory plant genes *NR* and *GS,* and N assimilation during host-symbiont interaction with different dry bean cultivars at different growth stages. The experiments tested the hypothesis that quantification of bacterial and plant marker genes could predict the amount of total N-assimilation as determined by legume plant genotypes. We studied the expression dynamics of dry bean *NR* and *GS* along with the *Rhizobium phaseoli nifH* gene in four dry bean commercial cultivars La Paz, Lariat, Windbreaker, and ND-307 at the third trifoliate (V_3) and late flowering (R_2) stages using robust gene specific primers that produced reproducible qPCR data. It was determined that ND-307 had higher *nifH* gene expression at both growth stages and fixed more N_2 than all other cultivars. At the R_2 stage, the *nifH* gene

expression was upregulated, whereas, *NR* and *GS* gene expression was downregulated compared to the V_3 stage. The amount of N_2 fixed by the cultivars was significantly correlated (r^2 = 0.82) with the relative normalized *nifH* gene expression, validating this molecular assay as a tool to screen N_2 fixation potential of dry bean. The downregulation of N-assimilatory genes at the later stages of growth indicated that SNF could not support plant N demand and supplemental N addition can benefit legume production.

Keywords: Dry bean, *Rhizobium*, symbiotic nitrogen fixation, nitrogen assimilation, *nifH*, nitrate reductase, glutamine synthetase

Introduction

Symbiotic Nitrogen Fixation (SNF), a significant component of the global nitrogen (N) cycle, is carried out by a class of prokaryotes (Bacteria and Archaea) known as 'diazotrophs'(Burns and Hardy, 1975; Ludden, 2001). About half of the N input in the global ecosystem is contributed by SNF (Vitousek et al., 1997). Although, N is abundant in the atmosphere, the dinitrogen (N_2) form is relatively inert and not reactive towards oxidation or reduction, thus, is unavailable to most organisms. Only diazotrophs (e.g. *Rhizobium, Azotobacter* etc.) can fix N_2 as free-living organisms or symbiotically in association with higher plants (Phillips, 1980; Howard and Rees, 1996).

Eukaryotic systems lack the genes to synthesize the nitrogenase enzyme complex that carries out reactions of N_2 fixation (Seefeldt et al., 2017). A class of higher plants in the family Fabaceae has the ability to engage in symbiotic relationships with *Rhizobia* and utilize the fixed N_2. Rhizobia can colonize legumes by forming nodules and live as a N_2 fixing symbiont (Peter et al., 1996; Hong et al., 2012). During SNF, atmospheric N_2 is reduced to ammonium (NH_4^+) or ammonia (NH_3) through reactions catalyzed by the two-component dinitrogenase-dinitrogenase

reductase complex that form the nitrogenase enzymes (Ludden, 2001). Nitrogenases are encoded by a set of operons that consists of structural genes (*nifH, D,* and *K*), regulatory genes (*nifL* and *A*), and a few supplementary genes in diazotrophic bacteria (Cheng, 2008). Nitrogenases have two main subunits: dinitrogenase (molybdenum-iron, Mo-Fe protein), which is the heterotetrameric core encoded by *nifD* and *nifK* and dinitrogenase reductase (Fe protein) encoded by *nifH* (Kim and Rees, 1994). The *nifH* gene is a universally accepted marker for SNF as it is found to be highly conserved among N_2 fixing organisms in natural environments and is widely used to study the ecological and evolutionary aspects of N_2 fixing bacteria (Izquierdo and Nüsslein, 2006; Farnelid et al., 2011).

About a quarter of the total N_2 fixation in the global ecosystem is contributed by rhizobia-legume symbiosis and the symbiotic compatibility between rhizobia and host legumes varies widely (Wang et al., 2018). Therefore, when studying N_2 fixation in this symbiotic system, the dynamics of *nifH* gene expression may provide a robust biomarker to identify host genotypes that are effective at inducing higher levels of N_2 fixation in association with their bacterial counterparts. In free-living diazotrophic communities, the *nifH* gene expression was found to be correlated with the amount of N_2 fixed and phenotypic expression (such as in *Azotobacter vinelandii* the correlation was found to be $r^2 = 0.72$ in soil and $r^2 = 0.84$ in liquid culture; Bürgmann et al., 2003); however, in symbiotic systems, this fact is yet to be discovered.

In a symbiotic system, there are mainly two sources of N available to the plant: available N from the soil as nitrate (NO_3^-) or NH_4^+, and NH_3 from SNF. The NO_3^- first must be reduced to NH_3 before it can be assimilated as organic nitrogenous compounds in the plant system (Crawford and Arnst, 1993). Reduction of NO_3^- is a two-step process: NO_3^- is first reduced to nitrite (NO_2^-) in the cytosol, a reaction catalyzed by the plant enzyme nitrate reductase (encoded

by *NR* gene), and then NO_2^- is reduced to NH_3 with the help of the enzyme nitrite reductase (Lam et al., 1996). NH_3 is assimilated into amino acids in stepwise reactions involving glutamine synthetase (GS), glutamate synthase (also known as glutaimine-2-oxoglutarate aminotransferase or GOGAT), and glutamate dehydrogenase (GDH) present as isoenzymes encoded by distinct genes (Lam et al., 1996; Mengel et al., 2001). Glutamine synthetase (GS; EC 6.3.1.2) is the key enzyme involved in the very first step of NH_3 assimilation, where it catalyzes the condensation of NH_3 and glutamate into glutamine with the help of another enzyme, glutamate synthase, in an ATP- dependent reaction (McGrath and Coruzzi, 1991; Cullimore and Bennett, 1992). Therefore, the genes that encode these enzymes involved in N-assimilation in the plant system are the genes that should be considered when studying gene expression to identify N-assimilation activity.

Dry bean, the second most important grain legume, is a relatively poor N_2 fixing legume plant (Tajini et al., 2008). Earlier studies reported that dry bean fixes approximately 35-40 kg of N ha^{-1}, while other grain legumes were reported to fix more than 70 kg of N ha^{-1} (Farid and Navabi, 2015). Some studies also reported that few cultivars of dry beans fixed more than 100 kg of N ha^{-1} (Rennie and Kemp, 1982). From earlier studies it was evident that there is large variation in the ability of dry bean genotypes to induce N_2 fixation, thus, there is potential to identify and utilize dry bean genotypes with higher N_2 fixing potential to facilitate genetic studies to understand the variability or polymorphisms within their genetic make-up that contribute to this positive agronomic attribute. Dry bean is one of the most important crops in the Red River Valley and North Dakota alone shares 39 percent of total dry bean produced in the USA. This information and genetic markers could then be utilized to select for higher N_2 fixing genotypes when developing dry bean cultivars in breeding programs.

The use of ^{15}N, a stable isotope of N, is the most reliable and accurate approach to quantify N_2 fixation and it has been extensively reviewed (Chalk, 1985; Witty et al., 1988). Among several ^{15}N-isotopic methods, the 'Isotope dilution technique' has high efficiency in discriminating the N_2 fixed from the atmosphere and the N taken up from the soil, though this technique is most accurate when the 'percent N derived from atmosphere' %Ndfa exceeds 70% and less suitable when N_2 fixation values are less than 30% (Danso, 1986; Barrie, 1991). A non-fixing reference plant is needed to estimate %Ndfa and both the fixing and reference plants should uptake N from the soil with same ^{15}N isotopic composition (Boddey et al., 1984). Moreover, ^{15}N enriched fertilizers for media enrichment as well as the analysis of enriched plant tissues in the Isotope Ratio Mass Spectroscope (IRMS) is very costly and sensitive to minimal variations.

Our study was primarily focused on determining if the plant genotype specific induction of rhizobial *nifH* gene expression could be indicative of the potential N_2 fixation potential and N assimilation during host-symbiont interactions. This outcome would also provide strong evidence that *nifH* gene expression correlates to the phenotypic expression in the symbiotic system i.e. amount of N_2 fixation. In addition, we were interested in studying the expression and regulation of the N assimilatory genes *NR* and *GS* during the symbiotic relationship, as there is a knowledge gap regarding the expression of these genes during the host-symbiont interaction. In this study, we report the first molecular evidence correlating *nifH* gene expression with phenotyp expression during the symbiotic relationship.

Materials and methods

Greenhouse condition and study design

Greenhouse experiments to study gene expression in dry bean cultivars were conducted at North Dakota State University (NDSU) during spring 2017. The greenhouse temperature was maintained at 30˚-35˚C during day and 19˚-23˚C during the night. A sixteen-hour light and eight-hour dark photoperiod was maintained in the greenhouse taking advantage of natural sunlight supplemented with high-pressure 60W artificial sodium artificial lighting.

Four commercial pinto bean cultivars, La Paz, Lariat, Windbreaker, and ND-307 were selected for this study (Table 1). Plants were grown in a 3:1 sand-vermiculite mixture in 2500 cm^3 closed bottom opaque pots. Pots were laid out in a completely randomized design (CRD) with factorial arrangement of four dry bean cultivars × two growth stages (V_3 and R_2) × two inoculation treatments (control and inoculated) × five replications.

Planting and fertilization

Non-treated (no fungicide treatment) dry bean seeds were washed with non-chlorinated water and pinched for better germination in the greenhouse. Seeds were then treated with a peat-based *Rhizobium* inoculant slurry following the manufacturer's instruction (N-Dure™, Verdesian), and subsequently air-dried for five minutes just before sowing. Four seeds were planted in each pot and one week after germination, seedlings were thinned to two seedlings per pot, and maintained until sampling. Plants were provided with a minimal amount of N (12.5 mg $plant^{-1}$) to force nodulation. All other nutrients were supplied through Hoagland's N-free solution (Sigma Aldrich®).

Table 5.1: Description, plant variety protection (PVP) numbers, and accession numbers of cultivars pinto cultivars selected for the study

Cultivars	Origin	Dry bean plant type	PVP #	Accession#
La Paz	Provita	USV (II)	200500219	PI 639241
Lariat	NDSU	USV (II)	200800305	PI 654383
Windbreaker	Seminis	UV (IIb)	200500105	PI 638525
ND-307	NDSU	UV (IIb)	200900009	PI 655297

UV: Upright Vine, USV: Upright short Vine, II: Upright – Indeterminate; IIb: Upright indeterminate, but prostrated/ decumbent in some environments; PVP #: Plant Variety Protection Number (USDA-AMS-ST-PVPO, 2017); Source ("U.S. National Plant Germplasm System,"; Osorno et al., 2013; USDA-AMS-ST-PVPO, 2017)

Sample collection

Plant samples were collected twice; first at the third trifoliate or V_3 stage, 35 days after planting (DAP) and second at the late flowering stage or R_2 (45 DAP). The V_3 stage is representative of the vegetative growth period and R_2 stage for reproductive growth. It is also known that nodules lose their ability to fix N_2 during pod filling which occurs during the R_5-R_6 stages (Franco et al., 1979; Flynn and Idowu, 2015). All samples were collected at the same time (between 2 pm and 4 pm) of the day to maintain the homogeneity among the samples since *NR* gene expression shows circadian rhythm (Cheng et al., 1991). The leaf samples were collected from the youngest trifoliate and only the middle leaflets were collected removing the petiole and leaf edges (~100 mg leaf tissue). Only pink-colored active nodules were collected with the help of sterilized forceps and about 30 nodules were collected from each plant. All the equipment and collected plant tissues were washed with 70 percent ethanol solution and RNaseZap™ RNase decontamination solution (Thermo Fisher Scientific™). The leaf and nodule tissue samples were collected in nuclease free centrifuge tubes and flash frozen in a liquid N_2 bath within two minutes after collection (half-life of RNA), which immediately moved from the liquid N_2 bath into -80˚C storage until RNA extraction was performed.

Total RNA extraction

Studying specific gene expressions in plant tissues needs a robust, highly efficient protocol to extract the total RNA from tissues, and this knowledge was lacking mainly for dry bean nodule and leaf tissues. In this study, total RNA was extracted from nodule and leaf tissue samples using the 'RNeasy Mini Kit' (Qiagen™) with modifications in the manufacturer's protocol (Qiagen, 2012). The new protocol was standardized and optimized after rigorous quality checks. RLT, RPE, and Lysis buffers were prepared following the manufacturer's instruction. 15 µl beta-Mercaptoethanol (β-ME) was added to 1 ml RLT buffer each time before use. Lysis buffer (400 µl) was used to homogenize plant tissue samples with disposable plastic pestles in 1.7 ml Eppendorf tube. For total RNA extraction from nodule tissues, 5-6 nodules (~25 mg) were used, whereas 25-30 mg of leaf tissue was used in the homogenization step. Homogenized samples were centrifuged for seven minutes for nodule tissues and four minutes for leaf tissues at maximum speed. After that ~350 µl of supernatant was carefully transferred to a 2 ml collection tube with a pipette to exclude cellular debris in the supernatant and 350 µl of 70 percent ethanol (made from Decon 200 Proof 100% pure ethanol) was added to it and mixed thoroughly with pipette. Subsequently, ~700 µl of the well-mixed sample was transferred to an RNeasy Mini spin column placed in 2 ml collection tube and centrifuged for 15 seconds at 8500 × g. The flow through was discarded and ~700 µl of buffer RW1 (Qiagen™) was added to the spin column and centrifuged for 15 seconds at 8500 × g. Thereafter, the flow through was discarded and ~500 µl of buffer RPE (Qiagen™) was added to the spin column and centrifuged for 15 seconds at 8500 × g. Again, the flow through was discarded and ~500 µl of buffer RPE was added to the spin column and centrifuged for two minutes at 8500 × g. Then the RNeasy Mini spin column was placed in another 2 ml collection tube and centrifuged at full speed for one minute. In the next

step, the RNeasy Mini spin column transferred to a new 1.5 ml collection tube supplied with the kit and 40 µl of warm (60˚C) ultrapure nuclease-free water (Ambion™) was added to the spin column and centrifuged for one minute at 8500 × g for elution. After this elution step, the eluent was passed through the spin column for a second time and centrifuged for 1 minute at 8500 × g. The second elution containing the total RNA was collected in centrifuge tubes and treated with DNase (Sigma-Aldrich®) to avoid genomic DNA contamination. The DNase-treated RNA then stored at -80˚C in 1.7 ml nuclease free collection tubes (Thermo Fisher Scientific™) until further analysis. RNA samples were visualized on 0.8% agarose gels stained with gel red (Biotium) to confirm the integrity of samples and quantified before storage using Qubit® 2.0 Fluorometer (Invitrogen™) with a high sensitivity or broad range RNA detection kit (Thermo Fisher Scientific™). Total RNA concentrations ranged between 40 and 200 ng μl^{-1} in nodules, 54 and 240 ng μl^{-1} in the leaves of inoculated plants, and between 45 and 352 ng μl^{-1} in the leaves of non-inoculated plants.

cDNA synthesis and primer designing

Total RNA (~160 ng) was used as template to synthesize cDNA using the 'GoScript™ Reverse Transcription System' (Promega™) following manufacturers protocol (RevTsc, 2010). Random hexamers, a mixture of oligonucleotides representing all possible sequence, were used instead of oligo-dT primers for cDNA synthesis as bacterial (prokaryote) RNA does not contain polyA tail. To maintain homogeneity in overall qRT-PCR reactions, random hexamers were used for cDNA synthesis from RNA. Resulting 20 µl cDNA was diluted 1:5 with 80 µl ultrapure nuclease-free water (Ambion™).

The primer pair reported in a recent *nifH* gene expression study in dry beans (Akter et al., 2014) were initially used in our study, but the primers did not amplify the specified portion of the

nifH gene even after gradient PCR. Other reported *nifH* primer pairs were either degenerate or not specific for rhizobia-dry bean symbiosis, but ultimately, did not amplify the target *nifH* gene (Bürgmann et al., 2003, 2004). Five new primers were designed to amplify different portions of *R. phaseoli nifH* gene and selected the most consistent primer pair after several gradient PCR amplification with these newly designed primers (Table 2). For plant *NR* and *GS* genes, no primers were reported earlier in the literatures for dry bean and the other primers reported in literatures were not specific to the dry bean *NR* and *GS* genes. In this literature, we report first ever gene specific, robust primers for dry bean *NR* and *GS* genes. There are several genes reported in literatures that could be used as reference genes in our study to normalize the gene expressions of the bacterial and plant genes. After several trials with different bacterial housekeeping genes such as *recA, dnaK, truA, dnaK* was selected as the reference gene to study *nifH* gene expression. For bean *NR GS, actin* was used as the reference gene in the qPCR study.

Use of degenerate primers in qPCR analysis of functional genes might introduce quantification bias, so degenerate primers were not used in our study (Gaby and Buckley, 2017). In this study, new, highly efficient, robust, gene specific primers were designed to reduce the possibility of off-target amplification, and gradient PCRs (Polymerase Chain reaction) were run using GoTaq® Polymerase (Promega™) to identify annealing temperatures that produced specific amplicons for each primer combination (Table 2). Amplicons of each primer pair for all the pinto cultivars at two time points were sequenced (GenScript) to confirm the target amplification and results are schematically shown in figure 2. The amplicons were also separated on 1% agarose gels supplemented with GelRed (Biotium) nucleic acid dye to check expected sizes.

Table 5.2: Primer sequences, their position in cDNA or genomic DNA, amplicon length, and annealing temperatures used for qPCR

Primer (Gene/ Protein ID)	Location (NCBI)	5'-3' nucleotide sequence	Position*	Amplicon Length (bp)	Annealing Temperature (˚C)
*nif*H (24297432)	NC_004041.2	F: GTGATGTCCGGCGAGATGAT	450	336	56.0
		R: GGTAATCGGGGTCGGAATGG	786		
dnaK (24299536)	NC_007761.1	F: GCCCGAAACATCTGACCCTG	860	377	55.7
		R: GGTGGTGTTGCGCTCGATCA	1236		
GS (CAA27631.1	X04001.1	F: GACACCACCGAGAAGGTCAT	40	113	58.0
		R: GGCAGCTCTGAAGGGTTCTTA	152		
NR (CAA37672.1)	X53603.1	F: AGCCAAAGAGATTGCGGTGA	2292	365	58.0
		R: AGTCGTAGACATGGCCGTTG	3333		
Actin (18615352)	KF033666.1	F: GAAGTTCTCTTCCAACCATCC	868	175	58.0
		R: TTTCCTTGCTCATTCTGTCCG	1042		

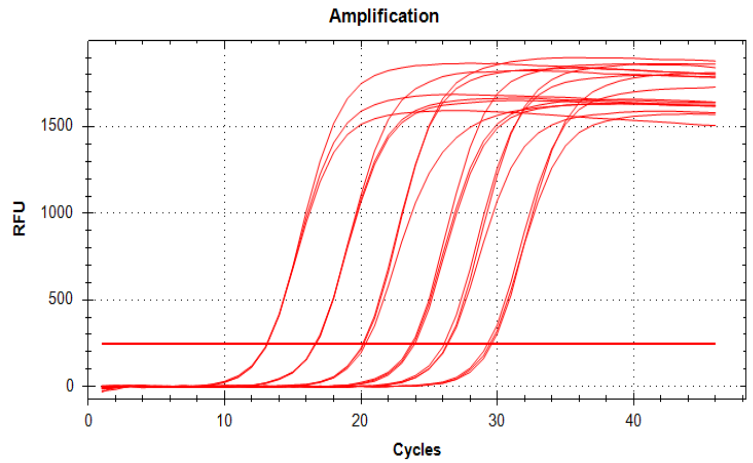
* Position of the primer nucleotide sequences in the corresponding gene sequence of specific organisms; *nifH* and *dnaK* in *Rhizobium phaseoli* and *Actin*, *NR* and *GS* in *Phaseolus vulgaris* L. F: Forward primer; R: Reverse Primer; bp: base pairs

qPCR analysis

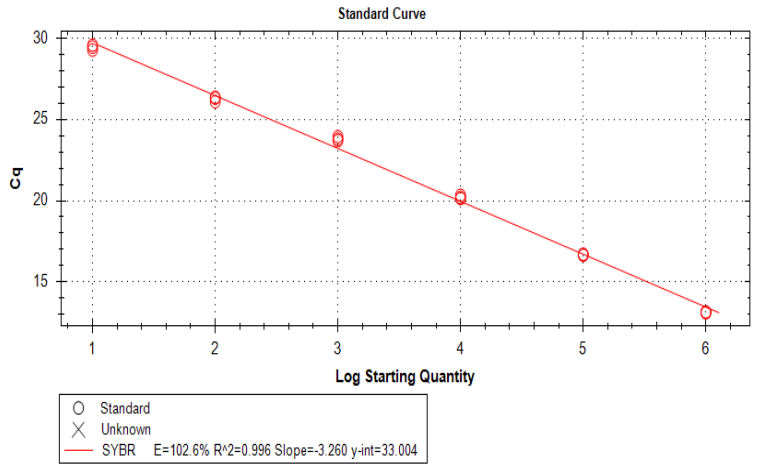
The qPCR (quantitative PCR) reactions were performed on three technical replicates of each of the three biological replicates using Bio-Rad Sso Advanced Universal SYBR® Green Supermix on a CFX-96 Real Time PCR detection system (Bio-Rad) (Solanki et al., 2016). A 10 µl qPCR reaction was prepared using 0.5 µl of 500 nM of each gene specific forward and reverse primer, 2 µl of diluted cDNA template, 0.1 µl polymerase, 2 µl Sso Advanced supermix, and 4.6 µl qPCR grade ultrapure water (Ambion®) in a hard-shell 96 well plate (BioRad) and sealed with Microseal 'B' PCR plate seal (BioRad). qPCR reactions were done following the thermal cycling protocol given below:

Polymerase activation and cDNA denaturation for 30 secs at 95°C, followed by 45 cycles of denaturation at 95°C for 15 sec and annealing at primer specific temperature for 30 sec. Then melt-curve was generated at the temperature from 65°C to 95°C with 0.5°C increment (2-5 sec per step).

For each qPCR plate analyzed, three negative controls (nuclease free water as template) were run for each primer pair. A ten-fold gene specific serial dilution from 15 $x10^{-1}$ pg (one pg = 10^{-12} g) to $15x10^{-6}$ pg was run in parallel to determine the efficiency of each qPCR run. Each qPCR plate contained samples of a specific plant tissue (root or leaves) at a specific growth stage only. Gene expression was normalized against the reference genes, *dnaK* for the *Rhizobium phaseoli nifH* gene, and *Actin* for *P. vulgaris* N-assimilatory *NR* and *GS* genes. Three biological replicates of each time point and treatment were analyzed using three technical replicates.



(a)



(b)

Figure 5.1: Representative standards (Actin) used for qRT-PCR: (a) Dilution series (15×10-1 to 15×10-6 picograms mL-1) used to produce standard curve; (b) Standard curve used to study gene expression and the calculated qPCR efficiency.
*RFU: Relative Fluorescence Units; *Cq is the quantification cycle (baseline-corrected) at which the amplification curve crosses some arbitrary threshold value (where the curvature of the amplification curve is maximal)

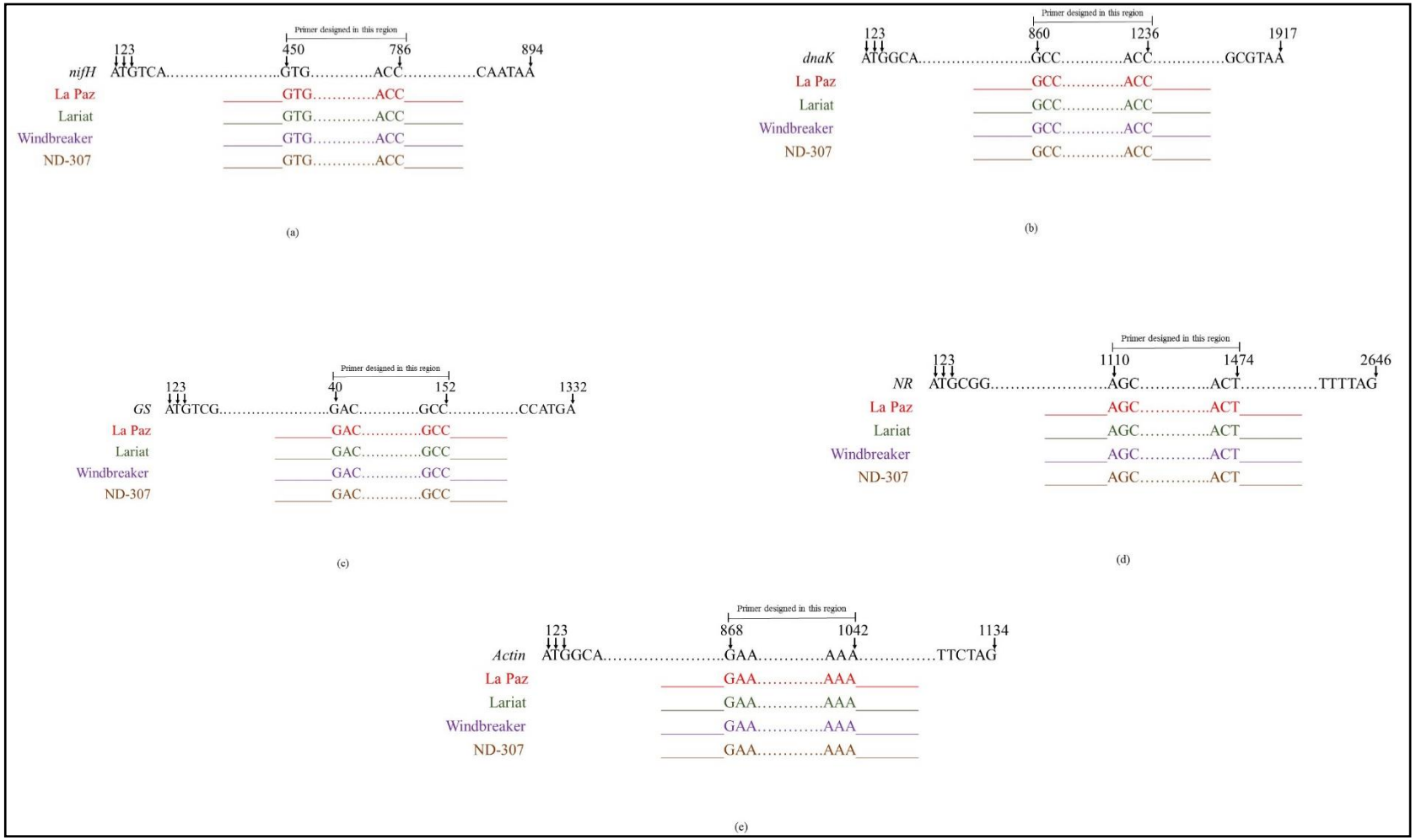


Figure 5.2: Diagrammatic representation of reference sequence from NCBI for each gene, a) *nifH*, b) *dnaK*, c) *GS*, d) *NR*, and e) *Actin*, used in qPCR along with the region utilized for gene specific primer designing and Sanger sequencing for dry bean (pinto) cultivar-specific amplification.

The digits denote the positioning of the DNA bases in the reference sequence. Legends: 1. '.' - conserved sequence, 2. '_____' - reference sequence, 3. ⊢————⊣ - Primer designing region

Relative expressions were calculated using comparative C_T method, where fold changes were calculated using the following equation and considering the assumptions (Schmittgen and Livak, 2008):

$$Fold\ Change = 2^{-\Delta\Delta C_T}$$

Gene expressions of two different samples were also compared using a modified form of this equation. The data analysis was carried out in 'Bio-Rad CFX Manager 3.1' software and Microsoft Excel. For all the studies, the values for cultivar La Paz at V_3 growth stage was utilized to determine the relative expression.

Sample preparation and estimation of N_2 fixation

At physiological maturity, fresh plant samples were weighed, and then dried at 55-60˚C for four days to prepare them for SNF estimation. $K^{15}NO_3$ was used as an N source to enrich the plant tissue with ^{15}N for estimation of SNF using the 'Isotope Dilution Technique'. Dried plant shoot tissues were ground and passed through a 0.02 mm screen, and approximately 3 mg of the ground samples were encapsulated into tin (Sn) capsules. Encapsulated samples were packed in 96-well trays, and sent to UC Davis Stable Isotope facility. Samples were analyzed for isotopic ratio (i.e. ^{15}N: ^{14}N) using a continuous flow Isotope Ratio Mass Spectrometer (PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 IRMS). Percent N derived from atmosphere (%Ndfa) was used as an indicator of SNF and was calculated from 'atom% ^{15}N' values of plant samples using the following formula (McAuliffe et al., 1958):

$$\%Ndfa = \left(1 - \frac{atom\%\ 15_N\ excess\ in\ N_2 - fixing\ plant}{atom\%\ 15_N\ excess\ in\ reference\ plant}\right) \times 100$$

The 'atom % excess ^{15}N' is the ^{15}N enrichment above the background (the value is 0.3663%). A non-nodulating dry bean white navy mutant line, 'R99', was used as the non-fixing

reference (Park and Buttery, 2006). The ^{15}N atom percent excess of these plant samples was used to calculate the %Ndfa values of the cultivars under investigation.

Results

Validation of primers and reference genes

The primers used in this study are robust and efficient, thus can be used in experiments with high precision. The *nifH* primer pair reported produces a *R. phaseoli* specific amplicon that can be used efficiently in future studies. The primers reported for *NR* and *GS* genes are also specific for dry bean. The primer pair that we developed to produce the dry bean *GS* gene specific amplicon amplifies the *GS* gene that is expressed in all the plant parts (Gebhardt et al., 1986), including leaves and nodules as we were interested in expression dynamics of *GS* in all tissues in order to better understand N assimilation.

Actin was used as a reference gene to normalize plant gene expression as use of *Actin* as a reference housekeeping gene in qPCR experiments is well reported in plants (Bashan et al., 2014; Bao et al., 2016; Li et al., 2016; Andrade et al., 2017). To study bacterial *nifH* gene expression, *dnaK* was selected as the reference gene. Throughout qPCR trials with *dnaK*, it showed constant Cq values (the number of qPCR cycles at which the baseline-corrected amplification curve crosses an arbitrary threshold value) for all the samples at a specific time point and specific concentration, a critical parameter to select a reference gene. Also, in earlier literatures, *dnaK* was reported to be used as a reference gene to study expression of *Rhizobium* and *Bradyrhizobium* genes, as this gene is highly conserved among different species of rhizobia (Stępkowski et al., 2003; Menna et al., 2009; Mnasri et al., 2012).

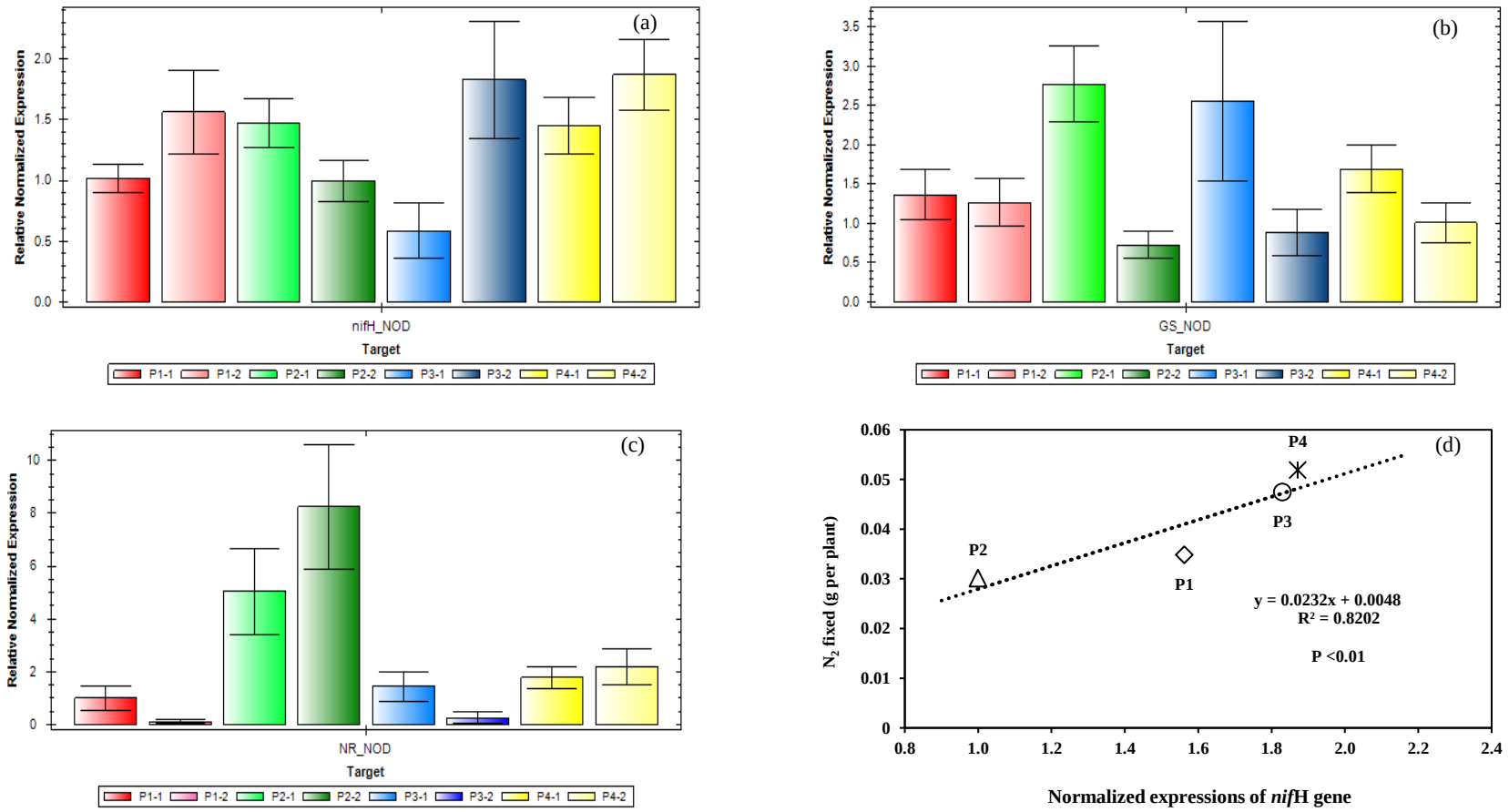


Figure 5.3: Relative normalized expressions of (a) *nifH*, (b) *GS* and (c) *NR* genes in root-nodules of four commercial pinto bean cultivars (La Paz, Lariat, Windbreaker, and ND-307) at two different growth stages (V_3 and R_2); (d) Linear regression model between mean amount of N_2 fixed (g N_2 per plant) by four cultivars and corresponding normalized expressions of *nifH* gene in their root-nodules at the R_2 stage; Values (bars) with overlapped standard error bars are not statistically significantly different ($P<0.01$); *nifH* gene expression was normalized against *dnaK* gene; *GS* and *NR* gene expression was normalized against the *Actin* gene; P_1: La Paz, P_2: Lariat, P_3: Windbreaker, and P_4: ND-307; 1 denotes V_3 stage and 2 denotes R_2 stage

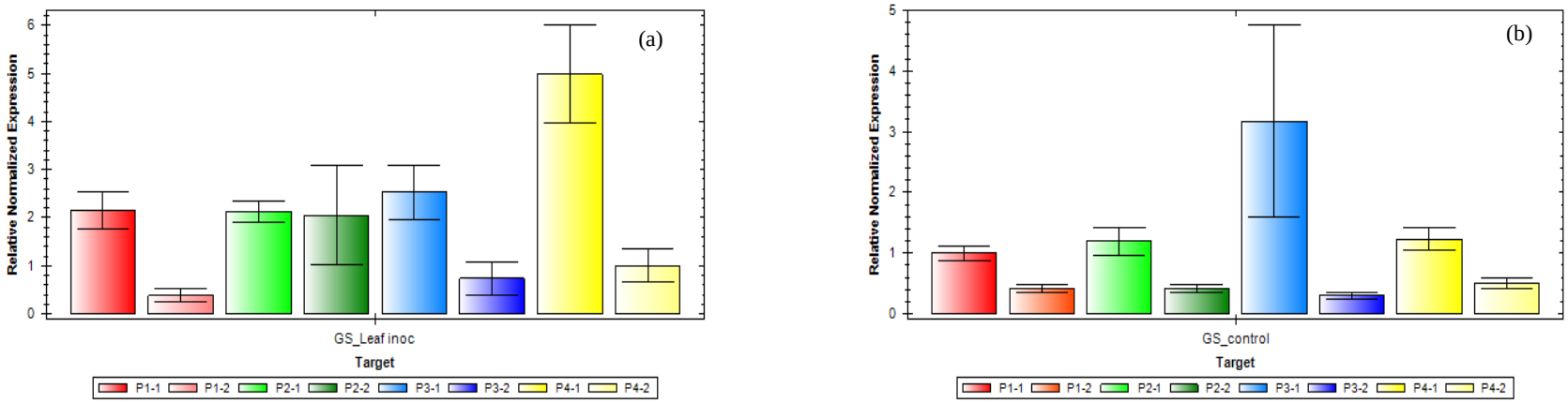


Figure 5.4: Relative normalized expression levels of the *GS* gene in the leaves of (a) Rhizobia inoculated plants and (b) control (non-inoculated) plants of four commercial pinto bean cultivars (La Paz, Lariat, Windbreaker, and ND-307) at two different growth stages (V_3 and R_2); *Values (bars) with overlapped standard error bars are not statistically significantly different ($P<0.01$); Gene expression was normalized against the *Actin* gene; P_1: La Paz, P_2: Lariat, P_3: Windbreaker, and P_4: ND-307; 1 denotes V_3 stage and 2 denotes R_2 stage

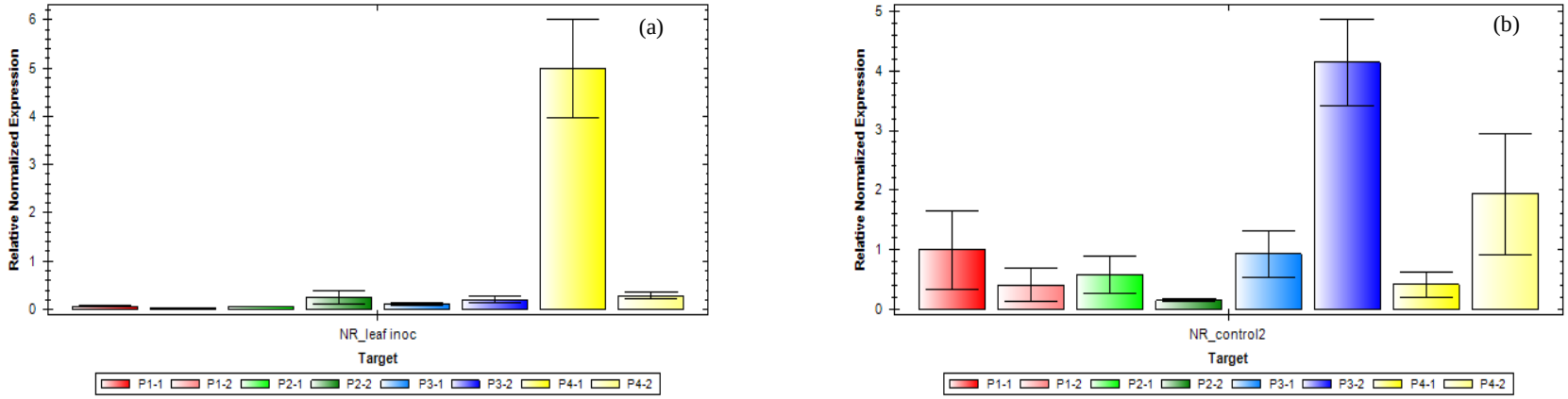


Figure 5.5: Relative normalized expressions of the *NR* gene in the leaves of (a) Rhizobia inoculated plants and (b) control (non-inoculated) plants of four commercial pinto bean cultivars (La Paz, Lariat, Windbreaker, and ND-307) at two different growth stages (V_3 and R_2); Values (bars) with overlapped standard error bars are not statistically significantly different ($P<0.01$); Gene expression was normalized against the *Actin* gene; P_1: La Paz, P_2: Lariat, P_3: Windbreaker, and P_4: ND-307; 1 denotes V_3 stage and 2 denotes R_2 stage

nifH gene expression in nodule tissue

All the experimental cultivars showed variable normalized *nifH* gene expression (Fig. 3a). ND-307 maintained constant high relative normalized bacterial *nifH* gene expression at both growth stages (i.e. V_3 and R_2), indicating a higher SNF activity compared to the other tested cultivars (Fig. 3a). Lariat had a higher relative normalized *nifH* gene expression level at the V_3 stage, followed by La Paz and Windbreaker. Conversely, Windbreaker had the higher relative normalized *nifH* gene expression followed by La Paz and Lariat at the R_2 growth stage (Fig. 3a). The relative normalized *nifH* gene expression was upregulated 1.5 and 3.1 folds in La Paz and Windbreaker, respectively, but downregulated 1.47 folds in Lariat at the R_2 stage compared to the V_3 stage for each cultivar (Fig. 3a). There was no change in relative normalized *nifH* gene expression for ND-307 between the two growth stages under investigation.

NR gene expression

In nodule tissues, relative normalized *NR* gene expression was found to be significantly variable among the four dry bean cultivars at both the V_3 and R_2 growth stages (Fig. 3c). At both growth stages, the highest *NR* gene expression levels were found in Lariat, and all other cultivars showed significantly lower *NR* gene expression. The other three experimental cultivars showed similar *NR* gene expression levels with ND-307 having the second highest *NR* gene expression, followed by Windbreaker, and La Paz at both the growth stages (Fig. 3c). Between the two growth stage assayed the relative normalized *NR* gene expression in nodules of the experimental bean cultivars La Paz and Windbreaker were significantly different. The *NR* gene expression was downregulated in nodule tissues of La Paz (8.2 fold) and Windbreaker (5.8 fold) at the R_2 stage compared to V_3 stage, and no significant change in gene expression was found in the other two cultivars (Fig. 3c).

In inoculated leaf tissue, relative normalized *NR* gene expression was found significantly variable among the cultivars at V_3 stage, but not at R_2 stage (Fig. 5a). ND-307 had significantly higher *NR* gene expression than the other three cultivars at V_3 stage, while the other three cultivars had statistically similar *NR* gene expression levels (Fig. 5a). Significant variation was found in the relative normalized *NR* gene expression levels in the inoculated leaves of the cultivars between the V_3 and R_2 growth stages (Fig. 5a). The *NR* gene expression was downregulated 2.9 and 14 folds in La Paz and ND-307, respectively; while upregulated in Lariat (4.5 fold) at the R_2 stage as compared to the V_3 stage (Fig. 5a).

In the non-inoculated leaves, statistically significant relative normalized *NR* gene expression among cultivars was found at the R_2 stage; highest *NR* gene expression was found in Windbreaker, followed by ND-307, La Paz, and Lariat (Fig. 5b). Relative normalized *NR* gene expression in the non-inoculated leaves was upregulated significantly at R_2 stage compared to V_3 stage (Fig. 5b). The gene expression was upregulated 4.5 folds for both Windbreaker and ND-307; but there was no change in gene expression for La Paz and Lariat (Fig. 5b).

_GS_ gene expression

Significant variation in relative normalized *GS* gene expression in the nodule tissue was found among the dry bean cultivars at both growth stages (Fig. 3b). Lariat showed the highest relative normalized *GS* gene expression followed by Windbreaker, ND-307 and La Paz at the V_3 stage, while, La Paz exhibited the highest relative normalized *GS* gene expression levels at the R_2 stage, followed by ND-307, Windbreaker, and Lariat (Fig. 3b). Downregulation of the *GS* gene expression was found in nodule tissue of the dry bean cultivars at the R_2 stage (Fig. 3b). The *GS* gene was downregulated in Lariat (3.8 fold), Windbreaker (2.9 fold), and ND-307 (1.7

fold) at the R_2 stage compared to V_3 stage, while there was no change in nodule *GS* gene expression in La Paz (Fig. 3b).

Relative normalized *GS* gene expression levels in the inoculated leaves varied significantly among the cultivars at both the V_3 and R_2 growth stages, (Fig. 4a). ND-307 had the highest relative normalized *GS* gene expression level at the V_3 stage, followed by Windbreaker, La Paz, and Lariat; while at R_2 stage, Lariat had the highest *GS* gene expression, followed by ND-307, Windbreaker, and La Paz (Fig. 4a). The *GS* gene expression levels in the inoculated leaf tissue of the dry bean cultivars significantly varied between the two growth stages. The relative normalized *GS* gene expression was downregulated at R_2 stage compared to V_3 stage. The *GS* gene expression in inoculated leaf tissue was downregulated in La Paz (5.7 fold), Windbreaker (3.5 fold), and ND-307 (5.0 fold) at the R_2 stage compared to V_3 stage; no statistically significant change in gene expression was found in Lariat between the two growth stages (Fig. 4a).

Significant variation in relative normalized *GS* gene expression in the uninoculated control leaves of dry bean cultivars was found at V_3 growth stage, but no significant variation in gene expression was found among dry bean cultivars at R_2 stage (Fig. 4b). Windbreaker had significantly higher *GS* gene expression in the control leaf tissue than the other three dry bean cultivars at the V_3 stage. *GS* gene expression was downregulated in the control leaves of all the cultivars at R_2 stage compared to V_3 stage (Fig. 4b). The *GS* gene expression in control leaves was downregulated 10.7 folds in Windbreaker, 2.9 folds in Lariat, and 2.4 folds in both Windbreaker and La Paz (Fig. 4b).

Discussion

In our experiments, different dry bean cultivars in symbiotic associations with *R. phaseoli* showed differences in relative normalized *nifH* gene expression as reported earlier (Knauth et al., 2005). It was also found that cultivars with higher *nifH* gene expression also fixed higher amount of N_2. Similar findings were also reported in earlier studies on N_2 fixing diazotrophic cyanobacterial communities (Vitousek et al., 1997; Warshan et al., 2016; Zehr et al., 2007). We have found significant linear correlation ($R^2 = 0.82$) between the relatively normalized expressions of the rhizobia *nifH* gene at the R_2 stage and the amount of N_2 fixed (g N_2 per plant) for all the four experimental cultivars in this study (Fig. 3d). Similar positive correlation was reported in studies on diazotrophic communities (Bürgmann et al., 2003; Thaweenut et al., 2011; Turk-Kubo et al., 2012). This outcome strongly supports our hypothesis that cultivars that induce higher *nifH* gene expression in their rhizobia symbiont will fix higher amounts of atmospheric N_2 than the cultivar showing lower gene expression. Our results provide the first molecular evidence for the plant host genotypic specific induced expression of the rhizobia *nifH* gene correlating with the phenotypic fixation of higher amounts of atmospheric N_2 in a legume-*Rhizobia* symbiotic system.

Plants absorb the available N from growth media at early growth stages and the reduced supply of N causes the downregulation of N-assimilatory genes. The regulation of *NR* gene expression by different levels of NO_3^- is well reported in earlier literatures (Galangau et al., 1988; Mohr et al., 1992). It was reported that NO_3^- is essential for the induction of *NR* gene expression and evidence of induced upregulation and downregulation of the *NR* gene were reported as plants were supplied with additional NO_3^-s and starved, respectively (Galangau et al., 1988; de Borne Dorlhac et al., 1994; Wang, 2000). Other studies suggested plants without any

accessible NO_3^- source had no detectable or very low *NR* mRNA expression (Hoff et al., 1991, 1992). In our study, *NR* gene expression showed a similar pattern of regulations and the Cq values for *NR* were as high as 33. Plants were intentionally supplied with low amounts of inorganic N to force SNF, which in turn affected *NR* gene expression. It is obvious that plants had access to more N as NO_3^- from the growth media during the early growth stages, and as the plants grew, NO_3^- availability got depleted since further NO_3^- was not added. The NR enzyme catalyzes the NO_3^- reduction and needs NO_3^- in the plant systems as discussed earlier. Therefore, suggesting that the decline in the available NO_3^- in the growth media diminished NO_3^- uptake at the later R_2 growth stage. Thus, due to scarcity of the substrate, NO_3^-, the activity of NR enzyme decreased, and subsequently the *NR* expression was found lowered.

Similar gene expression patterns were observed for the *GS* gene expression compared to the *NR* gene expression pattern in all four cultivars. Higher *GS* gene expression was found at the earlier V_3 growth stage as compared later R_2 growth stage used in the experiments. There are several possible hypotheses to explain these observations. Earlier, it was reported that there was a large increase in GS enzyme activities during nodulation (Lara et al., 1983). The nodulation in dry bean generally starts 2-3 weeks after planting, although it depends on other factors including growth environment and genetic make-up. GS enzyme activity also reaches its peak within two weeks (Flynn and Idowu, 2015). The sampling for V_3 stage was also done during this period, and our outcome of higher GS gene expression at V_3 compared to R_2 supports the observations that GS enzyme activity is higher during peak nodulation.

The GS enzyme uses NH_4^+ as its substrate and assimilates the NH_4^+ in the form of glutamine. The scarcity of NH_4^+ due to decreased activity of NR resulted in the decreased activity of GS. In other words, *GS* gene expression followed the downregulation pattern of the

NR gene expression at the R_2 stage compared to the V_3 stage. Due to continued unavailability of the substrate, NH_4^+, the *GS* gene was also shut down at later stages of growth.

The *nifH* gene expression was increased in the later R_2 growth stage, and one possible explanation is that during the plant-symbiont interaction the host can induce *nifH* gene expression in a plant genotype specific manner when another available source of N, NO_3^- are depleted. These data suggest that to meet its physiological N requirement the plants are able to induce SNF activity of the *Rhizobia* bacteria. At this stage, plants were also preparing for seed development, which requires the elevated synthesis of amino acids, and thus, more N is required for assimilation. However, at this stage SNF as the only source of N was not adequate to provide the required amount of NH_3 for assimilation into glutamine by the GS enzyme resulting in the suppression of *GS* gene expression. This outcome suggests that in the symbiotic systems with dry bean as the legume partner plant, addition of N through other sources is necessary to meet the N requirement at the later stages of growth, i.e. late vegetative to early reproductive stage. A conceptual model was prepared to connect all the findings of our experiments for better understanding of the gene expression dynamics in relation to the dry bean genotypes, crop growth stages, availability of N from growth media (Figure 6). Though further studies are needed to identify the exact growth stage (or time point) when the additional N source should be provided to the plant.

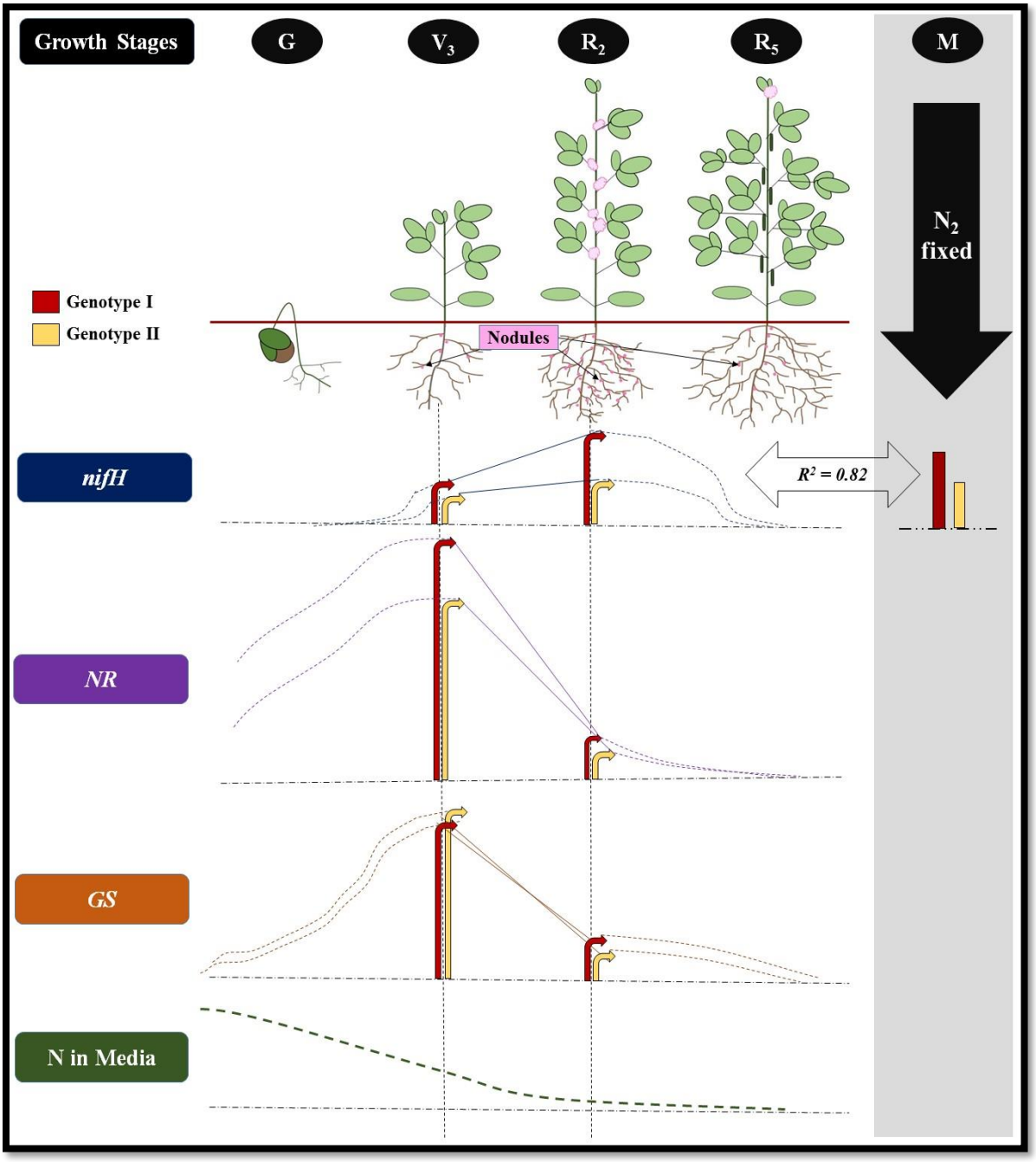


Figure 5.6: A conceptual model depicting estimated and predicted expression dynamics of the *Rhizobium phaseoli nifH* gene and dry bean (*Phaseolus vulgaris* L.) *NR* and *GS* genes at different growth stages (G: germination, V_3: third trifoliate stage, R_2: late flowering stage, R_5: pod-filling stage, M: maturity).
The expression levels shown are from two dry bean genotypes in relation to the available nitrogen from the growth media. The R^2 value shows the linear correlation between the relative normalized *nifH* gene expressions at late flowering stage and the amount of total nitrogen fixed.

Conclusion

We have developed a robust qRT-PCR method to show the expression profile of *NR* and *GS* gene in four dry bean cultivars along with nodule forming *R. phaseoli nifH* gene. This is the first ever study using molecular analysis to demonstrate that the higher N_2 fixing dry bean cultivars are also exhibiting higher *nifH* gene expression. The *nifH* gene expression was higher in the R_2 compared to V_3. Our analysis also concludes that at the initial growth stages of dry bean, *NR* and *GS* gene expressions are significantly higher due to higher availability of N from the growth media. However, at the later stages (R_2), significant reduction in expression was detected. The NR and GS enzyme activities are well reported in literatures, but molecular analysis-based on gene expression studies in relation to symbiont bacterial *nifH* gene expression were lacking. Our molecular assay will not only help to identify host genotypes that have the capability of inducing *nifH* gene expression correlated with higher SNF, but also facilitate the understanding of efficient timing of N fertilization. The ability to conduct robust molecular characterization of SNF during plant growth stages and will also increase our knowledge of the cross talk and signaling occurring during this complex symbiotic mechanism. Our findings regarding the correlation between the *nifH* gene expression and the amount of SNF should open a new avenue towards future investigations in symbiotic systems.

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CHAPTER VI: RESEARCH SUMMARY

Outcomes of our greenhouse experiment suggested that stable isotope dilution technique gave better estimate of N_2 fixation activity in dry bean cultivars compared to Ureide method and the new liquid inoculation helped in formation of nodules in dry bean roots. Thus, we used stable isotope dilution in our further research. The two-year long field experiment indicated that rhizobia inoculation provided *Rhizobium* strains that formed nodules in the roots of commercial dry bean cultivars from different market classes, but under field condition, when native rhizobia strains were present, the inoculants failed to improve N_2 fixation and seed yield. The new liquid inoculant resulted in higher N_2 fixation compared to peat inoculant in 2017, but fixation was higher in control. Dry bean seed yield was also significantly higher in control compared to inoculated plots, which indicated that inoculation adversely affecting the N_2 fixation, plant growth and metabolism. The most probable reasons were: firstly, the native *Rhizobium* strains were more efficient in symbiosis with dry bean cultivars under study compared to the inoculant strains; and secondly, the inoculant formulations were negatively affecting plant germination and thus, the crop stand and seed yield. To support our findings in the field experiment when expressions of nitrogen fixation (*nifH*) gene were studied, it was found that the gene expressions were highly positively correlated with the amount of N_2 fixed i.e. the phenotypic expression, indicating this to be genotype-dependent. This study also revealed the fact that when N_2 fixation activity decreased at late flowering stage, dry bean plants needed additional supply of N for optimum N metabolic activities as evident from N assimilatory gene expression. Furthermore, the *nifH* gene expressions can be used by the plant breeders to identify the dry bean cultivars with higher symbiotic efficiency, avoiding the costly, labor-intensive field trials.

APPENDIX: SUPPLEMENTARY TABLES

Table A1: Variations in mean plant dry matter yield and relative ureide N (%) of twelve dry bean cultivars under Pinto, Navy, Kidney and Black bean market classes at two growth stages

Market Class	Cultivar	%Ndfa		N_2 fixed (mg N $plant^{-1}$)
		V_3 stage	R_2 stage	
Kidney	Foxfire	58.4	71.2	32.7
	Redhawk	55.5	70.5	36.1
	Pink Panther	65.0	70.1	32.2
Pinto	La Paz	74.6	93.7	61.6
	Lariat	85.1	90.9	75.7
	Windbreaker	82.2	92.2	63.5
Navy	Ensign	84.4	95.3	68.8
	T9905	92.3	99.3	73.4
	Medalist	94.8	97.6	49.2
Black	Eclipse	52.2	75.8	41.8
	Loreto	47.5	73.9	42.2
	Zorro	46.8	76.8	44.6

Table A2: Monthly distribution of temperature (˚C) and rainfall (mm) during crop growing seasons in Prosper, ND (2016) and Felton, MN (2017)

Month	Prosper, ND (2016)		Felton, MN (2017)	
	Temperature (˚C)	Precipitation (mm)	Temperature (˚C)	Precipitation (mm)
May	13.2	16.8	6.0	27.2
June	19.1	87.9	11.5	87.9
July	21.2	50.0	14.2	50.6
August	18.1	52.6	11.4	18.5
September	15.3	152	9.1	141
October	7.5	6.86	1.1	8.89

Table A3: Variations in mean percent nitrogen derived from atmosphere (%Ndfa) at third trifoliate (V_3) stage among different inoculation treatments at Prosper, ND in 2016 and at Felton, MN in 2017

Inoculation	%Ndfa (V_3)	
	2016	2017
Control	78.1a (2.5)	53.2a (3.8)
Liquid	66.9b (3.2)	44.7b (3.1)
Peat	75.7ab (2.3)	54.3a (3.2)

Standard errors are given in parenthesis below mean values only for the significant effects; Means followed by same lowercase letter(s) are not significantly different ($P < 0.05$) among the inoculation treatments

Table A4: Variations in mean dry matter yield (kg ha-1) among different inoculation treatments at Prosper, ND in 2016 and at Felton, MN in 2017

Inoculation	Dry Matter Yield (kg ha^{-1})	
	2016	2017
Control	1221	2081a (143)
Liquid	1051	1771a (160)
Peat	1037	1542b (168)

Standard errors are given in parenthesis below mean values only for the significant effects; Means followed by same lowercase letter(s) are not significantly different ($P < 0.05$) among the inoculation treatments

Table A5: Variations in mean seed yield (kg ha^{-1}) among different inoculation treatments at Prosper, ND in 2016 and at Felton, MN in 2017

Inoculation	Seed Yield (kg ha^{-1})	
	2016	2017
Control	1540a (110)	1724a (84.5)
Liquid	1110b (114)	1563b (117)
Peat	1165b (178)	1657ab (94.1)

Standard errors are given in parenthesis below mean values only for the significant effects; Means followed by same lowercase letter(s) are not significantly different ($P < 0.05$) among the inoculation treatments

Table A6: Variations in mean percent nitrogen derived from atmosphere (%Ndfa) at third trifoliate (V_3) and seed yield (kg ha^{-1}) among kidney cultivars at Felton, MN in 2017

Kidney Cultivars	%Ndfa (V_3)	Seed yield (kg ha^{-1})
Foxfire	43.8b (3.0)	1235b (80.9)
Montcalm	59.1a (2.5)	1775a (57.3)
Pink Panther	43.2b (1.7)	1413b (44.6)
Redhawk	22.7c (4.3)	760c (106)

Standard errors are given in parenthesis below mean values only for the significant effects; Means followed by same lowercase letter(s) are not significantly different ($P < 0.05$) among the cultivars within a specific market class

Table A7: Effect of interactions between market classes and inoculation treatments on variations in mean seed yield (kg ha^{-1}) at Prosper, ND in 2016 and Felton, MN in 2017

Market Class	Seed Yield (kg ha^{-1})					
	2016			2017		
	Control	Liquid	Peat	Control	Liquid	Peat
Kidney	1360aB (163)	710bB (99.4)	516cB (103)	1423B (97.7)	1166B (150)	1298B (101)
Pinto	1720A (123)	1510A (113)	1814A (201)	2025A (52.0)	1959A (58.6)	2016A (48.1)

Standard errors are given in parenthesis below mean values only for the significant effects; Means followed by same lowercase letter(s) are not significantly different ($P < 0.05$) among different inoculation treatments for a specific market class in a specific year; Means followed by same uppercase letter are not significantly different ($P < 0.05$) between kidney and pinto market classes under a specific inoculation treatment in a specific year

Table A8: Effect of interactions between cultivars within market classes and inoculation treatments on variations in mean percent nitrogen derived from atmosphere (%Ndfa) at third trifoliate (V_3) stage at Felton, MN in 2017

Market Class	Cultivar	%Ndfa (V_3)		
		Control	Liquid	Peat
Kidney	Foxfire	52.7A (3.5)	43.0A (0.9)	35.6B (2.8)
	Montcalm	63.2A (5.1)	53.7A (1.5)	60.3A (2.2)
	Pink Panther	46.9A (1.0)	44.3A (1.7)	38.5AB (2.6)
	Redhawk	21.6abB (1.4)	9.6bB (1.4)	36.8aB (4.8)
Pinto	La Paz	84.5aA (2.7)	49.3b (1.8)	71.6a (7.0)
	Lariat	55.0B (4.4)	45.4	66.2
	ND-307	60.9B (5.8)	52.4	64.8
	Windbreaker	40.6B (3.2)	59.8	60.9

Standard errors are given in parenthesis below mean values only for the significant effects; Means followed by same lowercase letter(s) are not significantly different ($P < 0.05$) among different inoculation treatments for a specific cultivar; Means followed by same uppercase letter(s) are not significantly different ($P < 0.05$) among different cultivars within a specific market class under a specific inoculation treatment

Table A9: Effect of interactions between kidney cultivars and inoculation treatments on variations in mean seed yield (kg ha^{-1}) at Felton, MN in 2017

Kidney Cultivar	Seed Yield (kg ha^{-1})		
	Control	Liquid	Peat
Foxfire	1440AB (116)	1085B (108)	1179BC (67.7)
Montcalm	1894A (96.6)	1647A (68.8)	1785A (44.8)
Pink Panther	1312B (35.1)	1558AB (52.8)	1371AB (29.0)
Redhawk	1046aB (56.4)	375bC (49.0)	858abC (69.8)

Standard errors are given in parenthesis below mean values only for the significant effects; Means followed by same lowercase letter(s) are not significantly different ($P < 0.05$) among different inoculation treatments for a specific cultivar; Means followed by same uppercase letter(s) are not significantly different ($P < 0.05$) among different cultivars within a specific market class under a specific inoculation treatment

Table A10: Effect of interactions between cultivars within market classes and inoculation treatments on variations in mean dry matter yield (kg ha^{-1}) after combined analysis

Market Class	Cultivar	Dry Matter Yield (kg ha^{-1})		
		Control	Liquid	Peat
Kidney	Foxfire	3321	3786	2270
	Montcalm	3787	4858	3452
	Pink Panther	3469	2502	2399
	Redhawk	3557	2174	2095
Pinto	La Paz	4448	2408	3176
	Lariat	4304	3903	3301
	ND-307	3109	2686	3586
	Windbreaker	4087	3570	4054

Table A11: Effect of interactions between cultivars within market classes and inoculation treatments on variations in mean N_2 fixed (kg ha^{-1}) after combined analysis

Market Class	Cultivar	N_2 fixed (kg ha^{-1})		
		Control	Liquid	Peat
Kidney	Foxfire	45.6	34.6	19.9
	Montcalm	39.9	44.1	50.3
	Pink Panther	43.6	25.5	20.1
	Redhawk	41.0	23.8	29.9
Pinto	La Paz	47.9	38.5	33.0
	Lariat	41.2	44.0	34.1
	ND-307	30.0	31.0	46.0
	Windbreaker	48.0	35.1	34.9