Ashok Shrawat · Adel Zayed
David A. Lightfoot *Editors*

Engineering Nitrogen Utilization in Crop Plants

Engineering Nitrogen Utilization in Crop Plants

Ashok Shrawat · Adel Zayed
David A. Lightfoot
Editors

Engineering Nitrogen Utilization in Crop Plants

Springer

Editors
Ashok Shrawat
Biotechnology
Monsanto Company
Chesterfield, MO
USA

David A. Lightfoot
Department of Plant, Soil and Agricultural
  Systems
Southern Illinois University
Carbondale, IL
USA

Adel Zayed
Biotechnology
Monsanto Company
Chesterfield, MO
USA

This book is dedicated to the speakers of our "Engineering Nitrogen Use Efficiency Workshop," which has been held every year since 2003 at the Plant and Animal Genomics Conference, San Diego, USA. This tome is planned to be the first of many volumes on this topic because NUE Workshop speakers excel every year.

Please contact us with presentations for future Workshops.

Thanks to all.
Ashok Shrawat, David A. Lightfoot, and Adel Zayed

Preface

This book celebrates 14 years of the "Nitrogen Use Efficiency Engineering" workshop at the Plant and Animal Genome Conference every January in San Diego, California. Each chapter was volunteered by a presenter.

Nitrogen is important. It an essential component in cellular physiology being scarce in bio-available forms. In contrast, oxygen, carbon, and hydrogen are much more bio-available. Nitrogen is present in numerous essential compounds including nucleoside phosphates and amino acids that form the building blocks of nucleic acids and proteins, respectively. In plants, nitrogen is used in large amounts in photosynthetic pigments, defense chemicals, and structural compounds. However, inorganic N is difficult to assimilate. Dinitrogen in the atmosphere is highly inert. Reduction to ammonium requires the energy of a lightning bolt, petrochemicals, or 12 ATP dephosphorylations per molecule within a nodule or other anaerobic environment. Global warming may increase the frequency of lightning storms and hence raise NO concentrations. Warmer, more stressed crops will require more nitrogen fertilizers to be applied and heavy rains will increase losses due to runoff.

Warmer days will cause more of the applied ammonium fertilizers to escape from the cell as ammonia gas. Photorespiration, increased by heat stress, releases tenfold more ammonium than is assimilated from the environment, and plants only re-assimilate $\sim 98\%$ of this. Consequently, a haze of ammonia gas is found floating above a photosynthetic canopy. That ammonia may be lost on the wind or returned to the plant or soil by rains or dew falls. Any improvements to these nitrogen cycles can have a massive positive impact on the efficiency of agriculture, reduce its carbon footprint, and over geological timescales reverse some of the anthropogenic contributors to global warming.

The assimilation of ammonium has a second major problem associated with it. Ammonia is assimilated releasing one acidic proton per molecule. There is enough flux to reduce the pH of even well-buffered soils to concentrations that inhibit plant growth, both directly, and by the release of toxic concentrations of micronutrients (Al and Mn in particular). Reduction within a nodule or other anaerobic environment compounds this problem by releasing two protons per ammonium produced. Soil acidification is a worldwide problem on a massive scale. Nitrates and nitrites

provide a solution to the acidification problem, as their reduction to ammonium absorbs 3–4 protons. So a pH-balanced fertilizer should theoretically be a four to one mixture of ammonium and nitrates. Nitrates and nitrites are the ions produced by those lightning bolts that provide about 10% of the world's reduced nitrogen each year. However, they are not without costs and problems. Nitrite is highly toxic to photosynthesis and respiration and so must be immediately reduced to ammonium. Plants produce massive amounts of nitrite reductase for this purpose. Nitrate is benign, easy to store and transport and consequently is the major form of inorganic N found in plants. However, plants still produce tenfold more nitrate reductase than is absolutely needed for assimilation, growth, and yield. Why? This remains unclear.

The major problem with nitrates and nitrates in the environment is that they are water soluble and so are rapidly leached from soils. So much is lost from agricultural soils, industrial activity, and human waste treatments that the world's rivers, lakes, and oceans are significantly fertilized. Algae are the microorganisms that benefit the most from this fertilizer. Unfortunately, they run low on other nutrients (P,K) and so produce toxins to kill other organisms to obtain the limiting nutrients through their decomposition. In addition, they absorb much of the waters' oxygen at night killing even toxin resistant aerobes. Finally they bloom, blocking the light needed for photosynthesis by submerged organisms. Millions of acres of oceans are affected.

The major problem with nitrates in the human diet is that they are metabolized to a potent carcinogen (nitrosamine) in the acid of the human stomach problems. High nitrate and so nitrosamine amounts in human diets are associated with many different cancers as well as fertility problems. However, nitrates are naturally excreted in human and animal saliva for the purpose of producing some nitrosamines in the gut. This is because the combination of acid and nitrosamine effectively kills many human and animal pathogens. *Helicobacter pylori* is one example. This microbe causes stomach ulcers that left untreated often become cancerous. *H. pylori* is endemic and became more abundant as lifestyles became more stressful. Consequently, several epidemiological studies found diets high in nitrate to be healthy in the 1990s and beyond, whereas before that they were significantly unhealthy. Clearly, then the healthiest option is a low nitrate diet and low stress lifestyle. *H. phylori* and like pathogens and the lesions they cause are better treated with drugs than nitrosamines.

Microbes in the soil take up the bulk of all applied fertilizers before the plant can. Ammonium can be assimilated or oxidized to nitrite, nitrate, nitrous oxide, or dinitrogen by microbial activities. Plants have to absorb N from microbes by force, using highly efficient enzymes, or by trade through symbiosis. During symbiosis, the microbes are provided with sugars in return for ammonium. The microbes may be free-living in the rhizosphere or housed in specialized structures such as nodules. Symbiotic microbes produce a variety of chemical signals to elicit the delivery of sugars from the plants, and these systems are ripe for manipulation by biotechnology approaches.

Because soil particles do not naturally have many N-containing minerals, and because N can be readily lost from the rooting environment, N is the nutrient element that most often limits plant growth and agricultural yields. As noted above, nitrogen is found in the environment in many forms and comprises about 80% of the Earth's atmosphere as triple-bonded nitrogen gas (N_2). However, this large fraction of N is not directly accessible by plants and must be bonded to one or more of three other essential nutrient elements including oxygen and/or hydrogen through N-fixation processes, and carbon through N-assimilation processes. Plants are able to absorb a little NH_3 from the atmosphere through stomata in leaves, but this is dependent upon air concentrations. The ions NO_3^- and NH_4^+ are the primary forms for uptake in by plants. The most abundant form that is available to the plant roots is NO_3^-, and the most abundant form in leaves is NH_4^+. The process of nitrification by soil bacteria readily converts fertilizer NH_4^+ to NO_3^-. Relative nitrogen uptake is also dependent on soil conditions. Ammonium uptake is favored by a neutral pH and NO_3^- uptake is favored by low pH. Nitrate also does not bind to the negatively charged soil particles; therefore, it is more freely available to plant roots, especially through mass flow of soil water than is NH_4^+, which binds to negatively charged soil particles and so moves primarily by diffusion. The assimilation of NH_4^+ by roots causes the rhizosphere to become acidic, while NO_3^- causes the rhizosphere to become more basic.

In conclusion, the assimilation of inorganic nitrogen is a key process in the productivity of all crop plants, and there are many steps at which metabolic improvements can be made. In future, the ability to provide active nodules to non-legumes may provide a new impetus for agriculture, biotechnology, and crop science. Making crop N in foods and feeds will be critical advances. Reducing N loss to air and water will be critical. Therefore, we editors and workshop organizers thank the contributors for their work, often a lifetime avocation that began as a vocation!

Chesterfield, USA   Ashok Shrawat
Chesterfield, USA   Adel Zayed
Carbondale, USA   David A. Lightfoot

Contents

Chapter 1
Developing Crop Varieties with Improved Nutrient-Use Efficiency

David A. Lightfoot

Environmental Issues

Nitrogen is an essential component in cellular physiology. Only oxygen, carbon, and hydrogen are more abundant (Marchner 1995; Andrews et al. 2004). Nitrogen is present in numerous essential compounds, including pigments, nucleoside phosphates, and amino acids, that underlie photosynthesis, nucleic acids, and proteins. In plants, nitrogen is used in the largest amounts in photosynthesis, pigments, defense chemicals, and structural compounds. However, inorganic N is difficult to assimilate. Dinitrogen in the atmosphere is highly inert. Reduction to ammonium requires the energy of a lightning bolt, petrochemicals, or 12 ATP dephosphorylations per molecule within a nodule or other anaerobic environment (Kaiser et al. 1998; Reid et al. 2011). Global warming may increase the frequency of lightning storms and hence raise NO concentrations. Warmer, more stressed crops will require more nitrogen fertilizers be applied and heavy rains will increase losses due to runoff.

Warmer days will cause more of the applied ammonium fertilizers to escape from the cell as ammonia gas (Lightfoot et al. 1999; 2001; 2007; 2008; 2009; 2010). Photorespiration, increased by heat stress, releases tenfold more ammonium than is assimilated from the environment, and plants only re-assimilate a portion of this. Consequently, a haze of ammonia gas is found floating above a photosynthetic canopy. That ammonia may be lost on the wind or returned to the plant or soil by rains or dew falls. Any improvements to these nitrogen cycles (Carvalho et al. 2011; Tercé-Laforgue et al. 2004a, b) can have a massive positive impact on the efficiency of agriculture, reduce its carbon footprint, and over geological timescales, reverse some of the anthropogenic contributors to global warming.

D. A. Lightfoot (✉)
Department of Plant, Soil and Agricultural Systems: Genomics and Biotechnology,
Center for Excellence, The Illinois Soybean Center: Southern Illinois University,
Carbondale, IL 62901, USA
e-mail: ga4082@siu.edu

The assimilation of ammonium has a second major problem associated with it Jahns et al. 1999; 2000; Koivunen et al. 2004a, b). Ammonia is assimilated releasing one acidic proton per molecule (Marchner 1995). There is enough flux to reduce the pH of even well-buffered soils to concentrations that inhibit plant growth, both directly and by the release of toxic concentrations of micronutrients (Al and Mn in particular). Reduction within a nodule or other anaerobic environment compounds this problem by releasing two protons per ammonium produced (Indrasumunar et al. 2011). Soil acidification is a worldwide problem on a massive scale.

Nitrates and nitrites provide a solution to the acidification problem, as their reduction to ammonium absorbs 3–4 protons (Marchner 1995). So a pH-balanced fertilizer should theoretically be a four-to-one mixture of ammonium and nitrates. Nitrates and nitrites are the ions produced by those lightning bolts that provide about 10% of the worlds reduced nitrogen each year. However, they are not without costs and problems. Nitrite is highly toxic to photosynthesis and respiration, and so must be immediately reduced to ammonium. Plants produce massive amounts of nitrite reductase for this purpose. Nitrate is benign, easy to store and transport, and consequently is the major form of inorganic N found in plants. However, plants still produce tenfold more nitrate reductase than is absolutely needed for assimilation, growth, and yield (Wang et al. 2000; Kleinhofs et al. 1980). Why this is, remains unclear.

The major problem with nitrates and nitrates in the environment is that they are water soluble and so are rapidly leached from soils (Moll et al. 1982; Lee and Nielsen 1987; David et al. 1997). So much is lost from agricultural soils, industrial activity and human waste treatments that the world's rivers, lakes, and oceans are significantly fertilized (Cherfas 1990; Burkholder et al. 1992). Algae are the micro-organisms that benefit the most from this fertilizer. Unfortunately, they run low on other nutrients (P, K) and so produce toxins to kill other organisms to obtain the limiting nutrients through their decomposition. In addition, they absorb much of the waters oxygen at night killing even toxin-resistant aerobes. Finally they bloom, blocking the light needed for photosynthesis by submerged organisms. Billions of acres of oceans are affected worldwide.

The major problem with nitrates in the human diet and saliva cycle is that they are metabolized to a potent carcinogen (nitrosamine) in the acid of the human stomach (Moller et al. 1990; Mirvish 1985; Duncan et al. 1998; Tannenbaum et al. 1978). Antioxidants in popcorn and tea can help reduce them. High nitrate and so nitrosamine amounts in human diets are associated with many different cancers as well as fertility problems. However, nitrates have uses; they are naturally excreted in human and animal saliva for the expressed purpose of producing some nitrosamines in the gut. This is because the combination of acid and nitrosamine effectively kills many human and animal pathogens. *Helicobacter pylori* is one example. This microbe causes stomach ulcers that left untreated often become cancerous. *H. pylori* is endemic and became more abundant as lifestyles became more stressful. Consequently, several epidemiological studies found diets high in nitrate to be healthy in the 1990s, and beyond, whereas before that, they were significantly unhealthy. Clearly, then the healthiest option is a low-nitrate diet and

low stress lifestyle. *H. pylori* and like pathogens and the lesions they cause are better treated with drugs than nitrosamines.

Microbes in the soil take up the bulk of all applied fertilizers before the plant can (Trenkel 1997; Cabello et al. 2004; Garcia-Teijeiro et al. 2009). Ammonium can be assimilated, directly by GS, or oxidized to nitrite, nitrate, nitrous oxide, or dinitrogen by microbial activities. Plants have to absorb N from microbes by force or trade, using highly efficient enzymes in force, and by trade through symbiosis [reviewed by (Indrasumunar et al. 2012)]. During symbiosis (endo- or ecto-), the microbes are provided with sugars in return for ammonium (Zhao et al. 2005). The microbes may be free-living in the rhizosphere (ecto-) or housed in specialized structures such as nodules (endo-). Symbiotic microbes produce a variety of chemical signals to elicit the delivery of sugars from the plants, and these systems are ripe for manipulation by biotechnology approaches. The humate industry appears to be manipulating the ecto-systems (Pracharoenwattana et al. 2010; Ohno et al. 2010; Lehmann and Kleber 2015; Taha and Osman 2017). The oleaginous carbon seems to be assimilated by microbes in return for nitrogen released to the plant

Plant Assimilations

Because soil particles do not naturally have many N containing minerals, and because N can be readily lost from the rooting environment, N is the nutrient element that most often limits plant growth and agricultural yields (Specht et al. 1999; Duvick 2005; Krouk et al. 2010). As noted above, nitrogen is found in the environment in many forms and comprises about 80% of the earth's atmosphere as triple bonded nitrogen gas (N_2). However, this large fraction of N is not directly accessible by plants and must be bonded to one or more of three other essential nutrient elements including oxygen and/or hydrogen through N-fixation processes, and carbon through N-assimilation processes (Marchner 1995). Plants are able to absorb a little NH_3 from the atmosphere through stomata in leaves, but this is dependent upon air concentrations. The ions NO_3^- and NH_4^+ are the primary forms for uptake in by plants. The most abundant form that is available to the plant roots is NO_3^- and the most abundant form in leaves is NH_4^+. The process of nitrification by soil bacteria readily converts fertilizer NH_4^+ to NO_3^- (Trenkel 1997). Relative nitrogen uptake is also dependent on soil conditions. Ammonium uptake is favored by a neutral pH and NO_3^- uptake is favored by low pH. Nitrate also does not bind to the negatively charged soil particles; therefore, it is more freely available to plant roots, especially through mass flow of soil water than is NH_4^+, which binds to negatively charged soil particles and so moves primarily by diffusion. As noted above, the assimilation of NH_4^+ by roots causes the rhizosphere to become acidic, while NO_3^- causes the rhizosphere to become more basic.

Nitrogen uptake and assimilation summates a series of vital processes controlling plants' growth and development (Lam et al. 2003). Nitrate, nitrite, and ammonium uptakes (and re-uptakes following losses) occur against massive

concentration gradients that require lots of energy to generate and maintain. In agriculture, plants are spaced sufficiently that they have an excess of captured light energy relative to the N and C supplies. Transgenic plants over-expressing low affinity nitrate uptake transporter Nrt1 increased the constitutive but not the induced nitrate uptake. Equally, plants transgenic with Nrt2.1 the high affinity nitrate transporter increased nitrate influx under low N conditions (Fraisier et al. 2000).

Transgenic plants expressing an ammonium transporter increased NUE (Gupta et al. 2008, 2011). Glutamate receptors in transgenic plants provided better growth. Equally, the uptake of short peptides had positive effects. All these transport-associated phenotypes would be desirable in agricultural production systems directed toward greater efficiency and lower environmental impacts, and a stack of the three transgenes would be of interest.

Nitrate acquired in the roots can be reduced in the shoot or the root, or even stored in vacuoles in the root or shoot for later assimilation. However, nitrate must be reduced to a useable form. This occurs via a two-step process catalyzed by the enzymes nitrate reductase (NR) and nitrite reductase to form NH_4^+. Both enzymes are produced in massive excess compared to the flux needed through the pathway, and mutants that reduce their amounts by 90% do not have phenotypes (Kleinhofs et al. 1980). Equally, some transgenic plants over-expressing NR increased nitrate reduction but were not altered in phenotype (Crete et al. 1997; Curtis et al. 1999; Djannane et al. 2002; Lea et al. 2004). However, two studies of NR over-expressing transgenic plants did record altered phenotypes including increased biomass, reduced drought stress (Ferrario-Méry et al. 1998, 2002), and improved NUE and yield during N limitation (Loussaert et al. 2008). These phenotypes would be desirable in agricultural crops. The coupling of NR to photosynthesis should be possible by transformation of plants with a ferredoxin-dependent NR from cyanobacteria.

The ability to fix dinitrogen is restricted to the bacterial world, but is widespread among microbes (Ferguson and Indrasumunar 2011). Many different *nif* gene families exist, suggesting selection for variation has been favorable for species. The activity of *nif* requires an anaerobic environment, so transferring the enzymes to plants will be difficult. To date, transgenics in this field are bacterial, as in hydrogenase enhanced microbes, or if plant, they are designed to improve the chances of nodule occupancy by improved bacterial strains. Strains that are most likely to set up nodule occupancy are rarely the most efficient nitrogen fixers. Plants also often fail to maintain effective nodules through flowering and pod set. Soybean and common bean for example have senescent nodules by flowering (Sinclair et al. 2007). Some species do have indeterminate nodules and it would be a valid goal of biotechnology to transfer this trait to major legume crops.

The N acquired as NH_4^+ does not require reduction upon uptake into the root, thus providing some energy savings to the plant over that of the NO_3^- form (Marchner 1995). However, it does require assimilation to avoid loss, and at high concentrations (>10 mM) toxicity to the plant occurs (Meyer et al. 1997; 2006; Godon et al. 1996). This can occur if a double application is made by inaccurate

GIS systems. Various studies have shown that under conditions of excessive NH_4^+ uptake, most plant species will transport this N source to the shoot, which is more sensitive to ammonium ions.

One important process to build key macromolecules in any living organism is the acquisition and utilization of inorganic forms of nitrogen during metabolism (Lea and Miflin 2011). Plants use amino acids as well as their precursors and catabolic products for important metabolic activities. Various other roles of amino acids include nitrogen storage and transport and the production of a very large number of secondary compounds including structural lignin compounds, light-absorbing pigments, phenolics and plant hormones. Plants convert the available inorganic nitrogen into organic compounds through the process of ammonium assimilation which occurs in plants by two main pathways. The first and primary pathway involves a reaction with glutamate to form glutamine which is catalyzed by glutamine synthetase (GS, EC 6.3.1.2) and requires an energy source of adenosine triphosphate (ATP). There are several isoenzymes of GS based on their location in the plant (Ortega et al. 2006). In the cytosol, GS1 is composed of 3–4 different subunits. There is only one isoform in the root plastids or shoot chloroplasts (GS2) with one subunit. Expressed in germinating seeds or in the vascular bundles of roots and shoots, the cytosolic form (GS1) produces glutamine for intracellular nitrogen transport. GS2 located in root plastids produces amide nitrogen for local consumption, while GS2 in the shoot chloroplasts re-assimilates photorespiratory ammonium (Lam et al. 2003). GS1 is encoded by a set of 3–6 paralogs in different crop species, so hetero-hexamers can form. However, the Kms hardly differ. Amino acid identity between GS1 isoforms is very high and is even similar to GS2. GS2 has a short peptide extension at the C-terminus that might be involved in regulation by phosphorylation. Alleles of the GS1- and GS2-encoding genes do exist that differ in their regulation. Alleles of GS appear to underlie QTL determining NUE and yield (Cañas et al. 2009, 2010). Transgenic analyses have been made of GS2 but not GS1. Among 12 studies in 9 plant species, the phenotypes reported included enhanced accumulation of N, growth under N starvation, herbicide (PPT) tolerance, leaf-soluble protein, ammonia, amino acids, and chlorophyll. Some genes and constructs decreased growth; salt, cold and drought tolerance; seed yield and amino acid content (Cai et al. 2009). Therefore, the use of GS transgenics in agriculture will be useful and desirable but only with careful attention to regulation and expression (Hemon et al. 1990; Coque et al. 2008 Seebaueret al. 2004; 2010).

The glutamine molecules produced by GS are used by a whole series of transaminases to produce the 20 protein amino acids and some nonprotein amino acids. Cardinal among the transaminases is the reaction catalyzed by glutamate synthase (GOGAT, EC 1.4.1.14 and 1.4.7.1) to form glutamate (Forde and Lea 2007). There are two common isoenzymes of GOGAT including a ferredoxin-dependent GOGAT (Fdx-GOGAT) and an NADH-dependent GOGAT (NADH–GOGAT). While both forms are plastidic, the Fdx-GOGAT enzyme is predominately found in photosynthetic organs and the NADH–GOGAT enzyme is found more in non-photosynthetic tissues such as in roots and the vascular bundles of developing

leaves (Lea and Miflin 2011). An NADPH-dependent GOGAT can be found in certain organs and in many bacteria. Plants transgenic with the NADH-dependent plant GOGAT have been reported. Phenotypes included enhanced grain filling, grain weight, total C and N content, and dry weight. Phenotypes were very similar to the benefits reported from alanine dehydrogenase and asparagine synthase (Good et al. 2007; Shrawat et al. 2008), suggesting the transaminases are acting on a common pathway.

The second pathway for ammonium assimilation also results in the formation of glutamate through a reversible reaction catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.2), with a lower energy requirement than GS/GOGAT. There are also at least two forms of GDH that occur in plants that include an NADH-dependent form found in mitochondria, and an NADPH-dependent form localized in the chloroplasts of photosynthetic organs. In addition, there are enzymes capable of aminating reactions that resemble GDH. GDHs present in plants serves as a link between carbon and nitrogen metabolism due to the ability to assimilate ammonium into glutamate or deaminate glutamate into 2-oxoglutarate and ammonium. However, due to the reversibility of this reaction, the assimilatory role of GDH is severely inhibited at low concentrations of ammonium. Additionally, GDH enzymes have a low affinity for ammonium compared with GS which further limits their assimilatory effectiveness. It has been suggested that the NAD-requiring form of GDH may be involved in carbon rather than nitrogen metabolism (Coruzzi and Bush 2001) with glutamate catabolism providing carbon skeletons for both the TCA cycle and the energy production during carbon or energy deficit. Alternate functions for GDH have also been proposed in which it has been assigned the role of re-assimilating excess ammonium, due to the limited ability of the GS/GOGAT cycle, during specific developmental stages (Limami et al. 1999; Loulakakis et al. 2002), such as during germination, seed set, and leaf senescence (Coruzzi and Zhou 2001. In contrast to plant GDHs, those found in microbes are very active in the assimilation of ammonium. Plants did not have the opportunity to incorporate this type of NADPH-dependent GDH because the bacterial lines that gave rise to chloroplasts do not contain gdhA genes. The few cyanobacteria with GDH activity have acquired genes by transgenesis or cellular fusions. Transgenic plants in six crop species have been produced that express gdhA genes from 3 microbes (Ameziane et al. 2000; Mungur et al. 2005; 2006; Abiko et al. 2010). Phenotypes in plants include increased biomass, water deficit tolerance, nutritional value, herbicide resistance, N assimilation, NUE, WUE, amino acid, and sugar content. GDH genes used in this way are being evaluated for commercialization (Nolte et al. 2004; 2009; 2016). One problem faced by this and the alanine dehydrogenase transgenics is a dependence on soil type for some of the beneficial effects. GDH seems to provide a growth advantage on silty–loam clay soils common in the southern Midwest of the USA. In contrast, the alanine dehydrogenase transgenics seem to work best on sandy soils. Combining the technologies or altering their regulation might provide stable beneficial effects in many soil types and locations.

A variety of other enzymes exist that are capable of aminating reactions. Each will be a candidate for over-expression in transgenic plants (Nelson et al. 2007; Castiglioni et al. 2008; Centtury et al. 2008; Goldman et al. 2009; Vidal et al. 2010). Phenylalanine ammonia lyase has been used in many transgenic plants. Equally, the enzymes of cyanide assimilations (cysteine metabolism) might be more active than previously thought and could be manipulated. Alteration of the enzymes of heme and chlorophyll biosynthesis might be tried again. The *E. coli hemA* gene was functional *but hemB* became insoluble in plant chloroplasts (unpublished).

In conclusion, the assimilation of inorganic nitrogen is a key process in the productivity of crop plants, and there are many steps at which metabolic improvements can be made (Pathak et al. 2008; Pennisi 2008). In future, the ability to provide active nodules or at least nitrogenases to non-legumes will provide an impetus for biotechnology (Harrigan et al. 2010; Valentine et al. 2011; Rubio et al. 2012; Wang et al. 2012). Nutritional value will be another breakthrough. Simple yield at the grain elevator worked well for 50 years, but now yield of milk and meat from feed has become a major new initiative, reducing waste as feces in the process. In addition, combining new breeding methods, new assays, genome editing, existing transgenes, and new promoters for their regulation will provide for new avenues in crop NUE improvement.

References

Abiko T, Wakayama M, Kawakami A, Obara M, Kisaka H, Miwa T, Aoki N, Ohsugi R (2010) Changes in nitrogen assimilation, metabolism, and growth in transgenic rice plants expressing a fungal NADP(H)-dependent glutamate dehydrogenase (*gdhA*). Planta 232(2):299–311

Ameziane R, Bernhard K, Lightfoot DA (2000) Expression of the bacterial *gdhA* encoding glutamate dehydrogenase in tobacco affects plant growth and development. Plant Soil 221:47–57

Andrews M, Lea PJ, Raven JA, Lindsey K (2004) Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. Ann Appl Biol 145:25–35

Burkholder JM, Noga EJ, Hobbs CH, Glasgow HB Jr (1992) New 'phantom' dinoflagellate is the causative agent of major estuarine fish kills. Nature 358:407–410

Cabello P, Roldán MD, Moreno-Vivián C (2004) Nitrate reduction and the nitrogen cycle in archaea. Microbiology 150:3527–3546

Cai H, Zhou Y, Xiao J, Li X, Zhang Q, Lian X (2009) Overexpressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice. Plant Cell Rep 28(3): 527–537

Cañas RA, Quilleré I, Christ A, Hirel B (2009) Nitrogen metabolism in the developing ear of maize (Zea mays): analysis of two lines contrasting in their mode of nitrogen management. New Phytol 184(2):340–352

Cañas RA, Quilleré I, Lea PJ, Hirel B (2010) Analysis of amino acid metabolism in the ear of maize mutants deficient in two cytosolic glutamine synthetase isoenzymes highlights the importance of asparagine for nitrogen translocation within sink organs. Plant Biotechnol J 8:966–978

Carvalho Jde F, Madgwick PJ, Powers SJ, Keys AJ, Lea PJ, Parry MA (2011) An engineered pathway for glyoxylate metabolism in tobacco plants aimed to avoid the release of ammonia in photorespiration. BMC Biotechnol 11:111–121

Castiglioni P et al (2008) Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiol 147:446–455

Century K, Reuber TL, Ratcliffe OJ (2008) Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products. Plant Physiol 147(1): 20–29

Cherfas J (1990) The fringe of the ocean—under siege from land. Science 248:163–165

Coruzzi G, Bush DR (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. Plant Physiol 125:61–64

Coque M, Martin A, Veyrieras JB, Hirel B, Gallais A (2008) Genetic variation for N-remobilization and post-silking N-uptake in a set of maize recombinant inbred lines. 3. QTL detection and coincidences. Theor Appl Genet 117(5): 729–747

Coruzzi GM, Zhou L (2001) Carbon and nitrogen sensing and signaling in plants, emerging 'matrix effects'. Curr Opp Plant Biol 4:247–253

Crete P, Caboche M, Meyer C (1997) Nitrite reductase expression is regulated at the post - transcriptional level by the nitrogen source in Nicotiana plumbaginifolia and Arabidopsis thaliana. Plant J 11:625–634

Curtis IS, Power JB, de Llat AAM (1999) Expression of chimeric nitrate reductase gene in transgenic lettuce reduces nitrate in leaves. Plant Cell Rep 18:889–896

David MB, Gentry LE, Kovacic DA, Smith KM (1997) Nitrogen balance in and export from an agricultural watershed. J Env Qual 26:1038–1048

Djannane S, Chauvin JE, Meyer C (2002) Glasshouse behavior of eight transgenic potato clones with a modified nitratereductase expression under two fertilization regimes. J Exp Bot 53:1037–1045

Duncan D, Dykhuisen R, Frazer A, MacKenzie H, Golden M, Benjamin N, Leifert C (1998) Human health effects of nitrate. Gut 42:334–340

Duvick DN (2005) The contribution of breeding to yield advances in maize (Zea mays L.). Adv Agron 86:83–145

Ferrario-Méry S, Valadier MH, Foyer C (1998) Over expression of nitrate reductase in tobacco delays drought—induced decreases in nitrate reductase activity and mRNA. Plant Physiol 117:293–302

Ferrario-Méry S, Valadier MH, Valadier MH, Godefroy N, Miallier D, Hirel B, Foyer CH, Suzuki A (2002) Diurnal changes in ammonia assimilation in transformed tobacco plants expressing ferredoxin—dependent glutamate synthase mRNA in the antisense orientation. Plant Sci 163:59–67

Ferguson BJ, Indrasumunar A (2011) Soybean nodulation and nitrogen fixation, Chapter 4. In: Hendricks BP (ed) Agricultural research updates, vol 1, ©2011 Nova Science Publishers, Inc. ISBN: 978-1-61324-292-6

Forde BG, Lea PJ (2007) Glutamate in plants: metabolism, regulation, and signaling. J Exp Bot 58(9):2339–2358

Fraisier V, Gojon A, Tillard P, Daniel-Vedele F (2000) Constitutive expression of a putative high affi nity nitrate transporter in Nicotiana plumbaginifolia: evidence for post transcriptional regulation by a reduced nitrogen source. Plant J 23:489–496

Garcia-Teijeiro R, Lightfoot DA, Hernandez JD (2009) Effect of a modified urea fertilizer on soil microbial populations around maize (Zea mays L.) roots. Commun Soil Sci Plant Anal 40:2152–2168

Godon C, Krapp A, Leydecker MT, Daniel-Vedele F, Caboche M (1996) Methylammonium-resistant mutants of Nicotiana plumbaginifolia are affected in nitrate transport. Mol Gen Genet 250(3):357–366

Goldman BS, Darveaux B, Cleveland J, Abad MS, Sayed M (2009) Genes and uses for plant improvement. United States Patent Application 2009/0070897

Good AG, Johnson SJ, DePauw MD, Carroll RT, Savidov N, Vidamir J, Lu Z, Taylor G, Stroeher V (2007) Engineering nitrogen use efficiency with alanine aminotransferase. Can J Bot 85:252–262

Gupta, Liu J, Dhugga KS, Simmons CR (2008) Manipulation of ammonium transporters (AMTs) to improve nitrogen use efficiency in higher plants. US Patent Application #12/045,098

Gupta, Liu J, Dhugga KS, Simmons CR (2011) Manipulation of ammonium transporters (AMTs) to improve nitrogen use efficiency in higher plants. US Patent Application 13/043,109

Harrigan GG, Ridley WP, Miller KD, Sorbet R, Riordan SG, Nemeth MA, Reeves W, Pester TA (2010) The forage and grain of MON 87460, a drought-tolerant corn hybrid, are compositionally equivalent to that of conventional corn. J Agric Food Chem 57:9754–9763

Hemon P, Robbins MP, Cullimore JV (1990) Targeting of glutamine synthetase to the mitochondria of transgenic tobacco. Plant Mol Biol 15:895–904

Indrasumunar A, Searle I, Lin M-H, Kereszt A, Men A, Carroll BJ, Gresshoff PM (2011) Nodulation factor receptor kinase 1a controls nodule organ number in soybean (*Glycine max* L. Merr). The Plant J. 59:61–68

Indrasumunar A, Kereszt A, Gresshoff PM (2012) Soybean nodulation factor receptor proteins, encoding nucleic acids and uses therefor. US Patent Application 2011/0231952

Jahns T, Kaltwasser H (2000) Mechanism of microbial degradation of slow release fertilizers. J Polym Environ 8:11–16

Jahns T, Schepp R, Siersdorfer C, Kaltwasser H (1999) Biodegradation of slow-release fertilizers (methyleneureas) in soil. J Environ Polym Degrad 7:75–82

Kaiser BN, Finnegan PM, Tyerman SD, Whitehead LF, Bergersen FJ, Day DA, Udvardi MK (1998) Characterization of an ammonium transport protein from the peribacteroid membrane of soybean nodules. Science 281:1202–1206

Kleinhofs A, Kuo T, Warner RL (1980) Characterization of nitrate reductase-deficient barley mutants. Mol Gen Genet 177:421–425

Koivunen M, Horwarth WR (2004a) Effect of management history and temperature on the mineralization of methylene urea in soil. Nutr Cycl Agro Ecosyst 68(1):25–35

Koivunen M, Morisseau C, Horwarth WR, Hammock BD (2004b) Isolation of a strain of *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) utilizing methylene urea (ureaformaldehyde) as nitrogen source. Can J Microbiol 50(3):167–174

Krouk G, Crawford NM, Coruzzi GM, Tsay YF (2010) Nitrate signaling: adaptation to fluctuating environments. Curr Opin Plant Biol 13:266–273

Lam HM, Wong P, Chan HK, Yam KM, Chow CM, Corruzi GM (2003) Overexpression of the ASN1 gene enhances nitrogen status in seeds of Arabidopsis. Plant Physiol 132:926–935

Lea US, ten Hoopen F, Provan F, Kaiser WM, Meyer C, Lillo C (2004) Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme in vivo and nitrite accumulation. Plant J 35: 566–573

Lea PJ, Miflin BJ (2011) Nitrogen assimilation and its relevance to crop improvement, chapter 1. In: Foyer C, Zhang H (eds) Nitrogen metabolism in plants in the post-genomic era. Annual plant reviews 42. Wiley Blackwell, New Delhi, India

Lehmann J, Kleber M (2015) The contentious nature of soil organic matter. Nature 528. https://doi.org/10.1038/nature16069

Lee LK, Nielsen EG (1987) The extent and costs of groundwater contamination by agriculture. J Soil Water Conserv 42:243–248

Lightfoot DA, Long LM, Vidal ME (1999) Plants containing the gdhA gene and methods of use thereof. US Patent #5,998,700

Lightfoot DA, Long LM, Vidal ME (2001) Plants containing the *gdhA* gene and methods of use thereof. US Patent #6,329,573

Lightfoot DA, Bernhardt K, Mungur R, Nolte S, Ameziane R, Colter A, Jones K, Iqbal MJ, Varsa EC, Young BG (2007) Improved drought tolerance of transgenic *Zea mays* plants that express the glutamate dehydrogenase gene (*gdhA*) of *E. coli*. Euphytica 156:106–115

Lightfoot DA (2008) Blue revolution brings risks and rewards. Science 321:771–772

Lightfoot DA (2009) Genes for use in improving nitrate use efficiency in crops, chapter 7. In Wood AJ , Jenks MA (eds) Genes for plant abiotic stress. Wiley Blackwell, New York, pp 167–182

Lightfoot DA and Fakhoury A (2010). Methods of using plants containing the *gdhA* gene: Aflatoxin reduction and fungal rot resistance. Patent Pending 2012/708,174

Limami A, Phillipson B, Ameziane R, Pernollet N, Jiang Q, Roy R, Deleens E, Chaumont-Bonnet M, Gresshoff PM, Hirel B (1999) Does root glutamine synthetase control plant biomass production in lotus japonicus L.? Planta 209(4):495–502

Loulakakis KA, Primikirios NI, Nikolantonakis MA, Roubelakis-Angelakis KA (2002) Immunocharacterization of Vitis vinifera L. ferredoxin-dependent glutamate synthase, and its spatial and temporal changes during leaf development. Planta 215:630–638

Loussaert DF, O'Neill D, Simmons CR, Wang H (2008) Nitrate reductases from red algae, compositions and methods of use thereof. United States Patent Application 20080313775

Marchner H (1995) Mineral nutrition of higher plants. Academic Press, London, UK

Meyer R (1997) A genetic analysis of response to ammonium and methylammonium in Arabidopsis thaliana. MS thesis, SIUC, Carbondale IL, pp 118

Meyer R, Yuan Z, Afzal J, Iqbal J, Zhu M, Garvey G, Lightfoot David A (2006) Identification of *Gsr1*: a locus inferred to regulate gene expression in response to exogenous glutamine. Euphytica 151:291–302

Mirvish SS (1985) Gastric cancer and salivary nitrate and nitrite. Nature 315:461–462

Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency to nitrogen utilization. Agron J 74:562–564

Moller H, Landt J, Pederson E, Jensen P, Autrup H, Jensen OM (1990) Endogenous nitrosation in relation to nitrate exposure from drinking water and diet in a Danish rural population. Cancer Res 49:3117–3121

Mungur R, Glass ADM, Goodenow DB, Lightfoot DA (2005) Metabolic fingerprinting in transgenic Nicotiana tabacum altered by the Escherichia coli glutamate dehydrogenase gene. J Biomed Biotech 2:198–214

Mungur R, Wood AJ, Lightfoot DA (2006) Water potential during water deficit in transgenic plants: Nicotiana tabacum altered by the Escherichia coli glutamate dehydrogenase gene. Plant Growth Regul 50:231–238

Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstrom DC, Bensen RJ, Castiglioni PP, Donnarummo MG et al (2007) Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci USA 104:16450–16455

Nolte SA, Young BG, Mungur R, Lightfoot DA (2004) The glutamate dehydrogenase gene gdhA increased the resistance of tobacco to glufosinate. Weed Res 44:335–339

Nolte SA (2009) Metabolic analysis of resistance to glufosinate in gdhA transgenic tobacco. PhD dissertation, PLB, SIUC, pp 244

Nolte SA, Young BG, Tolley L, Lightfoot DA (2016) Metabolic fingerprint of the resistance to glufosinate in tobacco transgenic with the glutamate dehydrogenase gene gdhA. Crop Sci 57:1–15

Ohno T, He ZQ, Sleighter RL, Honeycutt CW, Hatcher PG (2010) Ultrahigh resolution mass spectrometry and indicator species analysis to identify marker components of soil and plant biomass-derived organic matter fractions. Environ Sci Technol 44:8594–8600

Ortega JL, Moguel-Esponda S, Potenza C, Conklin CF, Quintana A, Sengupta-Gopalan C (2006) The 3'untranslated region of a soybean cytosolic glutamine synthetase (GS) affects transcript stability and protein accumulation in transgenic alfalfa. Plant J. 45:832–846

Pathak RR, Ahmad A, Lochab S, Raghuram N (2008) Molecular physiology of plant nitrogen use efficiency and biotechnological options for its enhancement. Curr Sci 94:1394–1403

Pennisi E (2008) The blue revolution, drop by drop, gene by gene. Science 320:171–173

Pracharoenwattana I, Zhou W, Keech O, Francisco PB, Udomchalothorn T, Tschoep H, Stitt M, Gibon Y, Smith SM (2010) Arabidopsis has a cytosolic fumarase required for the massive allocation of photosynthate into fumaric acid and for rapid plant growth on high nitrogen. Plant J. 62(5):785–795

Reid DE, Ferguson BJ, Hayashi S, Lin Y-H, Gresshoff PM (2011) Molecular mechanisms controlling legume autoregulation of nodulation. Annal Bot 108:789–795

Rubio-Wilhelmi Mdel M, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM (2012) Ammonium formation and assimilation in P(SARK)::IPT tobacco transgenic plants under low N. J Plant Physiol 169(2):157–162

Seebauer J, Moose SP, Fabbri B, Crossland L, Below FE (2004) Amino acid metabolism in young maize earshoots: implications for assimilate movement and nitrogen signaling. Plant Physiol 136:4326–4334

Seebauer JR, Singletary GW, Krumpelman PM, Ruffo ML, Below FE (2010) Relationship of source and sink in determining kernel composition of maize. J Exp Bot 61:511–519

Shrawat Ashok K, Carroll Rebecka T, DePauw Mary, Taylor Gregory J, Good Allen G (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. Plant Biotechnol J 6:722–732

Sinclair TR, Purcell LC, King CA, Sneller CH, Chen P, Vadez V (2007) Drought tolerance and yield increase of soybean resulting from improved symbiotic N fixation. Field Crops Res. 101:68–71

Specht JE, Hume DJ, Kumudini SV (1999) Soybean yield potential–a genetic and physiological perspective. Crop Sci 39:1560–1571

Taha SS, Osman AS (2017) Influence of potassium humate on biochemical and agronomic attributes of bean plants grown on saline soil. J Hortic Sci Biotechnol https://doi.org/10.1080/14620316.2017.1416960

Tannenbaum SR, Fett D, Young VR, Land PD, Bruce WR (1978) Nitrite and nitrate are formed by endogenous synthesis in the human intestine. Science 200:1487–1491

Tercé-Laforgue T, Dubois F, Ferrario-Méry S, Pou de Crecenzo M-A, Sangwan R, Hirel B (2004a) Glutamate dehydrogenase of tobacco is mainly induced in the cytosol of phloem companion cells when ammonia is provided either externally or released during photorespiration. Plant Physiol 136:4308–4317

Tercé-Laforgue T, Mäck G, Hirel B (2004b) New insights towards the function of glutamate dehydrogenase revealed during source-sink transition of tobacco (Nicotiana tabacum) plants grown under different nitrogen regimes. Physiol Plant 120:220–228

Trenkel ME (1997) Improving fertilizer use efficiency. Controlled-release and stabilized fertilizers in agriculture. International Fertilizer Industry Association, Paris

Valentine V, Benedito VA, Kang Y (2011) Legume nitrogen fixation and soil abiotic stress, chapter 9. In: Foyer C, Zhang H (eds) Nitrogen metabolism in plants in the post-genomic era. Annual plant reviews 42. Wiley Blackwell, New Delhi, India

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutiérrez RA (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis thaliana. Proc Natl Acad Sci U S A. 107:4477–4482

von Wiren N, Gazzarrini S, Gojon A, Frommer WB (2000) The molecular physiology of ammonium uptake and retrieval. Curr Opin Plant Biol 3(3):254–261

Wang R, Guegler K, LaBrie ST, Crawford NM (2000) Genomic analysis of a nutrient response in arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. Plant Cell 12(8):1491–1510

Wang X, Peng F, Li M, Yang L, Li GJ (2012) Expression of a heterologous SnRK1 in tomato increases carbon assimilation, nitrogen uptake and modifies fruit development. Plant Physiol 169(12):1173–1182

Zhao Y, Li W, Zhou Z, Wang L, Pan Y, Zhao L (2005) Dynamics of microbial community structure and cellulolytic activity in agricultural soil amended with two biofertilizers. Eur J Soil Biol 41:21–29

Part I
Molecular and Physiological Aspects of Nutrient Use Efficiency

Chapter 2
Improving Nitrogen Use Efficient in Crop Plants Using Biotechnology Approaches

Perrin H. Beatty and Allen G. Good

Introduction

Plants require a source of fixed, or biologically reactive nitrogen (N) to produce molecules such as nucleotide bases and amino acids, in order to make macromolecules like DNA and proteins that are then required for the genome, cellular structures and overall growth. Low or insufficient available N limits the plant growth and yield (both biomass and grain) of crop plants. Plants obtain fixed-N from the soil as ammonia, nitrate, urea, amino acids and peptides. Some plants, such as legumes and poplar trees, form a symbiosis with diazotrophic bacteria where they exchange ammonia from the diazotrophs for carbon (C) molecules and a protected living niche from the plant. Diazotrophic bacteria express a nitrogenase enzyme complex that allows them to reduce atmospheric N_2 to ammonia (NH_3) in a process called biological nitrogen fixation (BNF; Beatty et al. 2015). This enzyme complex has only been found expressed functionally in bacterial species. For centuries, organic N fertilizers (livestock and green manure) have been used to increase crop production. Since the commercialization of the Haber–Bosch process to synthesize ammonia from atmospheric N_2, synthetic N fertilizers have also been used to increase yields (Smil 2004). Synthetic nitrogen fertilizer use has increased by a factor of nine over the last 50 years, and with the predicted global population of 9 billion by 2050, plus the need to reduce hunger and malnutrition, nitrogen fertilizer use will need to increase in order to increase crop yields (Lassaletta et al. 2014). In 2012, the Food and Agriculture Organization of the United Nations (FAO) suggested that food production will need to increase by 60% between 2005/07 and 2050 to meet the

P. H. Beatty (✉)
Dept. Oncology, University of Alberta, Edmonton, AB T6G 2R7, Canada
e-mail: pbeatty@ualberta.ca

A. G. Good
Department of Biological Sciences, University of Alberta,
Edmonton, AB T6G 2E9, Canada

needs of 9 billion people. Reducing our global yield gaps to produce more food is essential; however, the estimate increase in N fertilizer application needed to do this is 45–73%. However, ramping up our agricultural outputs needs to be done as sustainable as possible, while also lowering N fertilizer application rates (from either organic or synthetic sources), so as to reduce or even eliminate environmental damage from N pollution.

Synthetic N fertilizers are costly to produce and expensive to buy and transport; therefore, they are mainly used by farmers in developed countries or countries with synthetic N fertilizer subsidies (Good and Beatty 2011a). Farmers in developing countries tend to not have the resources to buy or transport synthetic N fertilizer. In addition, these small-holder farmers are frequently growing crops on nutrient poor soils, leading them to produce crop yields far below the optimal yield which means they have little to no produce to take to market. This has resulted in a cycle of poverty because low yields do not allow an investment in fertilizers or improved crop management. The advent of Norman Borlaugh's green revolution of the 1960s, where crop plants were bred for high yields, allowed many farmers in developing countries in North and South America to break out of this poverty cycle by growing high yielding varieties (Borlaug 1972; Good and Beatty 2011a). However, these high yielding crop varieties were bred to use high levels of N fertilizer and so promoted non-sustainable agricultural practices such as excess N fertilizer application.

Crop plants tend to have low nitrogen use efficiency (NUE), taking up approximately 40–50% of the applied N. The rest of the applied N fertilizer that is unavailable to the plant enters the environment as N pollution (Good and Beatty 2011a; Galloway et al. 2014; Ng et al. 2016; Beatty and Wong 2017). Global crop NUE has decreased from the early 1960s when it was 68%, to the current value of 47%. This indicates that on a global scale, over half of the N fertilizer inputs to agriculture are lost to the environment. Therefore, unless our cropping systems can improve their N use efficiency in a substantial way, increasing N fertilizer inputs will only provide a low gain in crop yield with further N pollution to the environment (Lassaletta et al. 2014).

Excess N fertilizer can pollute the environment in many different ways, depending on the form of fixed-N in the fertilizer. Nitrate-N fertilizer is water soluble and so easily leaches into waterways, leading to drinking water contamination and proliferation of algal blooms that in turn cause dissolved oxygen levels to drop and subsequent loss of marine life and diversity. A classic example of water pollution in the USA is the 1.57 million metric tons of nitrogen (mainly as nitrate) that is spewed into the Gulf of Mexico from the Mississippi River (US Geological Survey; https://ks.water.usgs.gov/pubs/fact-sheets/fs.135-00.html), resulting in the formation of a hypoxic dead zone that can be seen from space. Nitrate left in the soil (not taken up by the plants) is chemically reduced by denitrifying soil bacteria to form nitrite (NO_2^-), nitric oxide (NO gas), nitrous oxide (N_2O gas) and ultimately, biologically inert N_2 (gas). The gaseous reduced N compounds are released into the atmosphere causing environmental damage. Nitric oxide is a precursor chemical that leads to tropospheric ozone pollution, and nitrous oxide is a greenhouse gas (GHG) with 296 times the global warming potential (GWP) of CO_2 plus it is an

ozone-depleting chemical (Galloway et al. 2004; Davidson et al. 2015). Seventeen per cent of global GHG emissions are from agriculture and a third of the agricultural GHG emissions are from N fertilizers (Strange et al. 2008). Ammonia N fertilizer is volated to gaseous ammonium (NH_4^+), especially in wet soils and eventually leads to acid rain and dust formation. Acid rain causes soil nutrient and mineral depletion and loss of microbial diversity and is a health hazard for humans and other animals (Erisman et al. 2008). As an additional example of the environmental costs of agriculture N, researchers in the UK recently conducted a life cycle assessment of the pollution sources from the production of a loaf of bread (Goucher et al. 2017). Following the production of the bread from growing the wheat to packaging and transporting the loaf showed that the ammonium nitrate fertilizer supplied to the wheat was directly linked to 48% of the GWP, 39% eutrophication potential and 42% of the human toxicity potential.

We cannot ignore the past, present and future anthropomorphic changes to the Earth's climate and how this will affect agriculture, food production, food security, freshwater availability, available arable land, biodiversity, human health and the environment. It has become obvious that we need to focus on improving the agricultural industry to make it more sustainable and environmentally friendly, even with the realization that we need to increase yields. Please refer to the publications listed here for further information on anthropomorphic changes and sustainable agriculture (Leip et al. 2014; Zhang et al. 2015; Haines et al. 2017).

Measuring NUE

Agronomists have shown that although crop yields may be increasing, the NUE of many crop varieties is declining, in part due to high to excessive use of N fertilizers (Lassaletta et al. 2014). If NUE is calculated based on applied N, rather than soil available N, then as the applied N levels increase the crop NUE declines, even with an increase in yield. This is largely what explains the overall decrease in global NUE, although now that trend has been reversed in a number of key developed countries (Lassaletta et al. 2014). An analysis of the 50 year trend (1961–2009) within 124 countries in crop yield and N fertilizer inputs including; organic plus synthetic fertilizer, biological nitrogen fixation and atmospheric deposits, showed that some countries have improved both their crop NUE and yield whereas other countries have not (Lassaletta et al. 2014). Interestingly, Lassaletta et al. (2014) also saw that countries with a higher proportion of their N inputs from BNF than from synthetic N fertilizer also had better NUE. Other researchers have pointed out that this alternative approach to N fertilizer, of using BNF as an N input, could also improve NUE. The growth of BNF-symbiotic plants, such as legumes, as an N-source is under-utilized globally, given that most countries only dedicate a few per cent of arable land to legume crops (Crews and Peoples 2004) (Table 2.1).

NUE has been defined in many different ways (Table 2.2; Good et al. 2004); however, it is basically a ratio of the harvested product (as grain or biomass) to the

Table 2.1 N-related genes can be grouped into two classes made of a total of six gene families. Adapted from McAllister et al. 2012 and Han et al. 2015

Gene families	Target genes used in biotechnology	Gene name	References
Class 1 Growth and development			
Signalling	G-protein γ subunit	DEP1	Sun et al. (2014)
	Mitogen-activated kinase	SMG1	Duan et al. (2014)
	SNF1-related kinase	SnRK	Wang et al. (2012)
	Early nodulin-like protein	ENOD	Bi et al. (2009)
Regulation; Transcription factors and small RNA	DNA binding one zinc finger	DoF1	Li et al. (2013)
	bHLH transcription factor	SAT1	Chiasson et al. (2014)
	Nuclear factor Y	NFY	Chen et al. (2015)
	NAM, ATAF1,2 and CUC2	NAC1, 2	Yang et al. (2015)
	NAC-a6	NAC005	Christiansen et al. (2016)
	F-box protein	APO	Terao et al. (2010)
	Arabidopsis nitrate regulated 1	ANR1	Zhang and Forde (1998)
	ATL31 UBI-ligase	Atl31	Sato et al. (2011)
	PII regulatory protein	GLB1	Hsieh et al. (1998)
Senescence	Cytokinin oxidase/dehydrogenase	CKX	Ashikari et al. (2005)
	Stay-green protein	SGR	Park et al. (2007)
Class 2 N metabolism pathways			
Transporters	Nitrate transporter	NRT	Tsay et al. (1993)
	Ammonium transporter	AMT, SAT1	Yuan et al. (2007)
	Lysine histidine transporter	LHT	Hirner et al. (2006)
	Hexose transporter	STP13	Schofield et al. (2009)
	Amino acid permease	AAP1	Rolletschek et al. (2005)

Table 2.1 (continued)

Gene families	Target genes used in biotechnology	Gene name	References
Amino acid biosynthesis	Alanine aminotransferase	*AlaAT*	Shrawat et al. (2008)
	Asparagine synthetase	*ASN*	Lam et al. (2003)
	Aspartate aminotransferase	*aspAT*	Ivanov et al. (2012)
	Asparaginase	*ASNase*	Zhou et al. (2009)
	Glutamate dehydrogenase	*GDH*	Abiko et al. (2010)
	Glutamine synthetase	*GS*	Brauer et al. (2011)
	Glutamate synthase	*GOGAT*	Tamura et al. (2011)
N-assimilation	Nitrate reductase	*NR*	Lea et al. (2006)
	Nitrite reductase	*NiR*	Takahashi et al. (2001)
C:N metabolism and storage	Rubisco small subunit	*Rubisco*	Masle et al. (1993)
	Ferredoxin NADP(H) reductase	*FNR*	Hanke et al. (2008)
	Isopentenyl transferase	*IPT*	Rubio-Wilhelmi et al. (2011)
	Cell wall invertase	*CIN*	Wang et al. (2008)

Table 2.2 Definitions, formula and inherent statistical considerations for NUE calculations

#	Term	Numerator	Denominator	Formula	Measurement	Statistical considerations
1	Nitrogen use efficiency (grain)	Yield	Ns	NUE = Gw/Ns	Grain yield	Ratio
2	Utilization efficiency (grain)	Yield	Nt	UtE = Gw/Nt	Grain yield	Ratio
3	Nitrogen use efficiency (biomass)	Yield (Biomass)	Nt	NUE = Sw/Nt	Biomass, feed, food	Ratio
4	Nitrogen use efficiency (grain)	Yield	Np	NUE = Gw/Np	Grain yield	Ratio
5	Agronomic Efficiency	Difference in yield	N supplied	AE = (GwF − GwC)/ N fert	Yield response	Numerator is difference, calculation a ratio
6	Physiological efficiency	Difference in yield	N supplied	PE = (GwF − GwC)/ Nfert − Ncon	Yield response	Numerator is difference, calculation a ratio
7	Efficiency of fertilizer uptake	N in plant	N supplied	UpE = Nt/Ns	Uptake of N by plant	Ratio, unaffected by total yield

Gw Grain weight
Ns Total N supplied to plant*
Nfert N Fertilizer applies (kg/ha)
Soil N SN
Total N − SN + Nfert
*In reality, Ns could be N applied, or total N available (N applied + soil N)