Controlling nitrogen over enrichment in agriculture

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# Summary

Nitrogen is one of the key ingredients that organisms require to live. Farmers have been using nitrogen fertilizers to increase their crop yields. The overuse of nitrogen fertilizer has been damaging the ecosystems that are surrounding agricultural areas. If too much nitrogen leaks into the surrounding water ways algal blooms can become harmful problems, with significant damage to the aquatic ecosystem as a result. In the nearby terrestrial ecosystems, an increase in nitrogen is also problematic as plant species that thrive in those conditions outcompete those that are not able to take advantage of the increased nitrogen levels. In order to combat these problems a decrease in nitrogen fertilization is required. These strategies are dependent on the knowledge of plant nitrogen uptake, as plants can take up nitrogen in several different forms which are all dependent on different uptake mechanisms.

Several studies have shown that often fertilizers are overused such that a decrease in application would not decrease the yield. In order for farmers to find out whether they have applied enough fertilizer they need to the ability to measure the nitrogen content in the crops that are growing in their fields. The measurement of sun induced fluorescence could allow farmers to do exactly that. By measuring the reflected light from the plants scientists have been able to accurately estimate the nitrogen content in plants at the canopy scale. If applied by farmers this would allow them to measure the nitrogen content of plants, potentially allowing them to only apply fertilizer when and where it is required.

The other solutions proposed in this review are based on genetical engineering. Researchers found the *zmm28* mutant in maize, called DP202216, to have a higher nitrogen uptake when compared to the wildtype maize strain. In this mutant a transcription factor called zmm28 is fused to a constitutive promoter. It remains unclear however what the molecular mechanism behind this increase is. Other researchers found an *Arabidopsis thaliana* mutant that reacts differently to nitrate deficiency stress then the wildtype. The wildtype shows reduced root growth under nitrate stress conditions, while the *NAC075* mutant found by these researchers does not. In these mutants the nac075 gene is mutated such that the protein cannot leave the cells where it is produced. The underlying molecular mechanism has been uncovered which makes it an interesting target for research in other species.

In order to actually lower the nitrogen input in the field a combination of several solutions might be the best option as they are not mutually exclusive. Using a transgenic crop similar to the mutants DP202216 or NAC075 could decrease the amount of nitrogen used in agriculture and thus reduce the severity of the problems that it causes. Farmers would also be able to measure the nitrogen content of these plants to even further decrease the amount of over fertilization.

# Abstract

Contemporary farming methods are based on fertilization of crops to reduce the limiting factor posed by some nutrients. Nitrogen is one of the most important compounds and is often over applied which causes over enrichment. This directly results in the loss of biodiversity in surrounding ecosystems as the nitrogen concentrations increase there, and it causes eutrophication of water bodies which in turn causes ecological damage to aquatic ecosystems. This review focusses on two types of solutions which could reduce over enrichment. The amount of nitrogen fertilizer applied to crops is in some cases too high, which causes unnecessary ecological damage. Measuring the amount of nitrogen that crops have been able to take up would prove beneficial in combating over enrichment. Recent research has shown that Sun Induced Fluorescence could be used as a measuring technique to estimate the leaf nitrogen content and the nitrogen use efficiency at the canopy scale. The importance of this discovery is that this would allow the use of this measuring technique in a real world agricultural setting. The second solution covered in this review is based on genetical engineering. As a lot is known about how nitrogen is taken up from the environment by plants, many genetic targets are available. Researchers found that the DP202216 hybrid in maize had improved nitrogen uptake when compared to wild type maize. As this constitutively overexpressing *zmm28* mutant was found by chance in a transgenics pipeline, not particularly much is known about the molecular mechanism which enables this increase in nitrogen uptake. But it could still be used as a potential maize variety which is able to thrive in lower nitrogen conditions. In contrast much more is known about the *NAC075* mutant which was found in an *Arabidopsis thaliana* mutant assay where this mutant showed increased root growth under low nitrate conditions when compared to the wild type. The molecular mechanisms behind this phenotype have also been uncovered as the phenotype is dependent on the intercellular transport of the NAC075 transcription factor. These are examples of traits that can be adapted to increase the nitrogen uptake efficiency of crops through the use of genetical engineering. To successfully decrease the amount of ecological damage to the environment due to over enrichment of nitrogen a combination of the mentioned solutions seems beneficial as they are not mutually exclusive.

# Introduction

The growing world population is dependent on ever increasing levels of food production. To keep up with this rising demand contemporary high efficiency farming methods are dependent on the use of fertilizers. Nitrogen is one of the elements on which life is based, as nitrogen is the fundamental element required for the formation of amino acids. This makes nitrogen one of the crucial nutrients for every living organism. Even though nitrogen is the most abundant element in the atmosphere where it resides in the form of N2, it is unsuitable for uptake for most organisms in this form.

Every living organism has a way to access nitrogen through the nitrogen cycle (as seen in figure 1). First, Nbecomes deposited into the soil. One of the pathways in which this happens is through soil residing nitrogen bacteria which have evolved the ability to fix nitrogen into the soil in the form of ammonia (NH3) and ammonium (NH4+). This is a very energetically costly process since N2 is a very stable compound due to the triple bond between the two N atoms. Due to this energy requirement, only a small selection of prokaryotes has the ability to fix nitrogen. Though nitrogen fixation in most environments occurs trough these prokaryotes, some abiotic fixation takes place during lightning strikes and in certain industrial processes such as the combustion of fossil fuels. The step in the nitrogen cycle is nitrification, which is also done through nitrifying soil residing bacteria. These bacteria, depending on the species are able to convert either ammonia (NH3) or ammonium (NH4+) into nitrite (NO2-). The nitrite is then catalyzed into NO3- by different soil bacteria. Plants are able to take up nitrate, nitrite, ammonia and ammonium and use their nitrogen content for the formation of amino acids. To complete the nitrogen cycle, denitrification is performed by yet another class of microorganism. In this process NO3- is converted back into N2. The denitrifying bacteria use nitrate as an electron acceptor, instead of oxygen. The ability to use nitrate is critical for these species allowing them to thrive in anaerobic environments such as waterlogged soils.

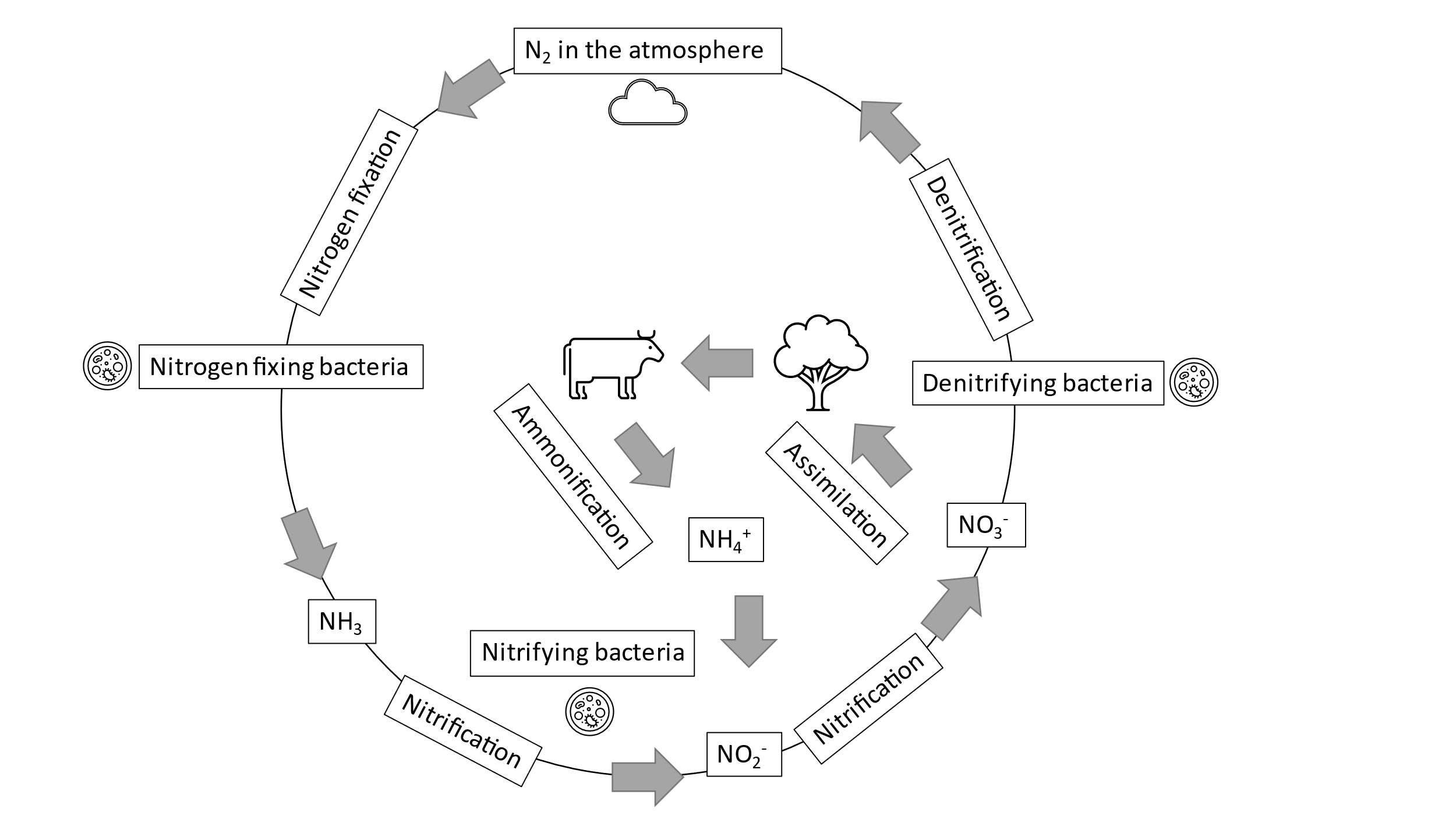


Figure 1: The nitrogen cycle

In the absence of human influence, the nitrogen cycle is a closed loop in most ecosystems. Depending on the geographical location combined with the ecology, an area can have either high or low amounts of naturally occurring nitrogen. An area with a high amount of nitrogen fixing bacteria will have a higher concentration of nitrogen available in the soil, and the reverse is also true. Because nitrogen is one of the limiting factors of growth, certain plant species have adapted to low nitrogen concentrations.

To maximize crop yield, farmers add commercial fertilizers to the crops in order stop nitrogen from being a limiting factor for yield. There are some major drawbacks to the overuse of these fertilizers, however. Nitrogen is one of the more expensive nutrients to supply and commercially available fertilizers are a large monetary investment in farming. This makes it that in economically weaker areas, farmers are not able to properly fertilize their crops with nitrogen, thus possibly decreasing their yields. Another major concern is the leaching of nitrogen from the field into the surrounding ecosystems. This gives rise to soil and water pollution.

Nitrogen application has a positive effect on many traits that are desirable in crop farming. This includes the actual crop yield quality (Bondada et al., 1996). Nitrogen fertilization also increases CO2 assimilation rate (Chen et al., 2010). Limited nitrogen availability has a major role in both stunted plant development and early leaf senescence, which directly leads to decreased crop production and crop quality (Dong et al., 2012).

## Problems caused by over enrichment of crops

An estimated 50-70% of nitrogen that is supplied to the soil is lost (Hodge et al., 2000). The lost nitrogen is either flushed into surrounding water systems or into the immediate surrounding ecosystem. An increase to the amount of nitrogen in an ecosystem can lead to nutrient saturation. Plant species in these ecosystems that have adapted to the naturally low nitrogen soil concentrations are outcompeted by species that have adapted to relatively higher concentrations. Especially when a certain critical load of nutrient deposition is reached. This results in a loss of biodiversity in this ecosystem. Biodiversity loss is a long term problem because the recovery of naturally occurring vegetation is a slow process even when the additional nitrogen input is halted (Nordin et al., 2005). Excessive nitrogen fertilizer application is a common problem, which has been shown to be highly counterproductive. High concentrations of nitrogen cause lower yields for a variety of crops, including fruit trees and fruity vegetables such as: tomato and cucumber (Erel et al., 2008; Stefanelli et al., 2010).

The nutrients that are flushed into the waterways can also cause problems, namely the eutrophication of water bodies. Similar to soil, water ecosystems also have a limited amount of available nitrogen when in the absence of human influence. The increased nutrient enrichment of these water bodies allows phytoplankton, algae and aquatic plant populations to increase dramatically. Nitrogen mostly causes eutrophication in saltwater systems. When freshwater systems drain into coastal ecosystems nitrogen becomes the dominant limiting factor (Ryther & Dunstan, 1971). While in lakes phosphate is the dominant limiting factor for eutrophication. This means that eutrophication cannot be controlled by reducing nitrogen input in lakes (Schindler et al., 2008). If phosphorus input is uncontrolled, nitrogen fixing cyanobacteria become prevalent, which are not limited by the nitrogen concentration in their environment. This mechanism allows lakes to still be highly eutrophic even in low nitrogen concentrations (Schindler, 1977). In absence of anthropogenic nutrient input eutrophication is a slow process in which nutrients accumulate (Elser et al., 2007). Anthropogenic eutrophication however is a much more rapid process where nutrients accumulate faster due to the amount of input nutrients. The forementioned 50-70% of lost nitrogen from fertilization thus attributes to the eutrophication and to harmful algal blooms. Degradation of these algae by bacteria can result in oxygen depletion of these aquatic ecosystems, in some cases causing significant ecological degradation in their water bodies (Sellner et al., 2003).

In order to decrease the total amount of nitrogen over enrichment in crop farming, a strategy is required. There are several ways of defining the plant’s efficiency with available nitrogen in the soil. N uptake efficiency (NUpE) is an established metric that is often used to display nitrogen management. For example: The amount of grain yield per unit of nitrogen. N Utilization efficiency (NUtE) addresses the yield produced per unit of nitrogen acquired by the plant. For example: The amount of grain yield per unit of taken up nitrogen. When these factors are combined it is called the N use efficiency (NUE) (Congreves et al., 2021). The end goal is to increase the NUE, such that less nitrogen fertilizer is required. This can thus be accomplished by increasing either the NUpE or the NUtE. Due to the large difference in pathways between the use and uptake of nitrogen, the focus of this review is placed solely on possible solutions to increase NUpE in crops. (Hirose, 2011; Moll et al., 1982)

## Nitrogen uptake and transportation mechanisms in plants

Soil nitrogen availability can be affected by factors such as temperature, wind, soil type, pH and precipitation (Maathuis, 2009). Plants have therefore adapted to take up nitrogen in the form they typically encounter in their ecosystem. Plants adapted to low pH and reducing soils, such as mature forests and tundra, are specialized in the uptake of ammonium. Plants adapted to higher pH and more aerobic soils have a preference to take up nitrate (Maathuis, 2009). The outer tissues of the plant root are responsible for nitrogen uptake as they are the parts of the plants that have physical access to the nitrogen compounds in the soil.

It is believed that the majority of plants have a symbiotic relationship with fungi in a mycorrhizal association. This symbiosis is dependent on the transfer of nutrients between both of the symbionts (Marschner & Dell, 1994). It is thought that the majority of nitrogen that is supplied to the plant is in the form of inorganic nitrogen compounds. The mycelium of the fungus has a large surface area which increases the amount of nitrogen it can take up. The nitrogen is taken up and then stored in the form of Arginine. The arginine is then broken down into inorganic nitrogen compounds close to the root where the plant is able to take these up (Marschner & Dell, 1994).

## Ammonia uptake and transport

As ammonia is readily converted into ammonium, high concentrations are only found near the surface in the atmosphere and close to emission sources such as industrial areas (Ferm, 1998). This is why ammonia is taken up mostly from the atmosphere through the leaves of higher plants. Ammonia is able to enter the stomata where it is dissolved in the water film of the mesophyll cells where it quickly coverts into ammonium (van Hove et al., 1988). Ammonia uptake flux increases linearly with the concentration of ammonia in the atmosphere (Van Hove et al., 1987) but is however dependent on the microclimate. This includes factors such as air temperature, humidity and air turbulence (van Hove et al., 1990). In roots no specific transportation mechanisms have been found as yet, which is probably because ammonia in soil is readily converted into ammonium. Although it has been speculated that at least some amount of nitrogen supplied to plants by the symbiotic relationship with fungi is in the form of ammonia (Chalot et al., 2006). The uptake of this supplied ammonia would then be taken up by the root through nonspecific channels such as passive diffusion through aquaammoniaporins (Loqué et al., 2005) or by voltage-dependent cation systems (Roberts & Tyerman, 2002). As in the leaves, the ammonia transported into the roots is quickly converted into ammonium in the presence of protons or when dissolved in water (Chalot et al., 2006).

## Ammonium uptake and transport

Like ammonia, ammonium is also taken up in the shoot parts of the plant. Ammonium accumulates on leaf surfaces trough both dry and wet deposition. It is thought that the uptake mechanism in shoots is similar to the uptake mechanism of ammonia as the ammonium is able to enter the stomata (Chevone et al., 1986) and uptake is possible either through diffusion or proton or cation exchange (Wilson, 1992).

While ammonium is less mobile then nitrate in the soil, it is still accessible by plants under most soil conditions. The ratio of nitrate to ammonium uptake by roots is heavily dependent on a large number of factors. The pH of the soil influences which nitrogen compound is more easily accessible by the roots (Vessey et al., 1990). Uptake of ammonium directly leads to acidification of the soil surrounding the roots, because ammonium is exchanged for a proton which is exuded by the roots (Bolan et al., 1991). As a side effect, competition in uptake between ammonium and different cations such as potassium takes place (Vale et al., 1987). This directly leads to potassium deficiency, when plants are provided with ammonium as their only nitrogen source. Several studies have shown that nitrogen deficiency induces increased nitrogen uptake capacity, which means that a signaling pathway of some sort is present, which has a stimulating effect on the uptake of nitrogen under certain conditions (von Wirén et al., 2000). Consequently, the genes linked to ammonium transport are a significant molecular engineering target to lessen nitrogen deficiency stress responses in plants.

In most soil conditions, ammonium is found at the micromolar range (R.J. Bijlsma & H. Lambers, 2000) the uptake of which is specifically facilitated by the ammonium transporter (*AMT*) gene family in *Arabidopsis* (Gazzarrini et al., 1999)*.* Many genes in this family have been cloned and have had their specific functionalities uncovered in different plant species, such as: maize (Gu et al., 2013), rice (Sonoda et al., 2003), tomato (Von Wirén et al., 2000) and Arabidopsis (Gazzarrini et al., 1999). In *Arabidopsis* there are a total of 6 AMT members which are grouped into AMT1 and AMT2 subtypes based on their sequence (von Wittgenstein et al., 2014). The AMT1 subgroup in *Arabidopsis,* consists of AMT1;1 to AMT1;5, which all show high affinity for ammonium (Yuan et al., 2007). The AMT1;1, AMT1;2, AMT1;3 and AMT1;5 genes are all expressed in the roots, and their expression levels are increased during nitrogen deficiency stress (Yuan et al., 2007). The AMT1;1, AMT1;3 and AMT1;5 genes are mainly expressed in cell layers that are in close proximity to the soil in both the roots and root hairs, while AMT1;2 is mostly expressed in the endodermal cells (Yuan et al., 2007). AMT1;1, AMT1;2 and AMT1;3 proteins show an additive contribution to ammonium uptake when knocked out, up to a total reduction of 90% (Yuan et al., 2007). This information tells us that the AMT1 family is a crucial part of the ammonium uptake mechanism in *Arabidopsis*, which might make the gene family an interesting target for molecular engineering. Different plant species have a differing amount of genes in the AMT1 family with the genome of rice containing at least 10 AMT1 genes (Sonoda et al., 2003). The number of AMT1 genes in the different plant species has been linked to their expression levels under low nitrogen stress and no stress situations (Gazzarrini et al., 1999). The AMT1 transportation mechanism is described as a ammonium uniporter that transport ammonium along the electrochemical gradient (Ludewig et al., 2002).

As mentioned above ammonia taken up by the shoots quickly reaches an equilibrium with ammonium in the water film of the mesophyll tissue. This equilibrium is strongly dominated by ammonium after which is it is assimilated. Ammonium taken up by the roots from soil however is metabolized locally. Assimilation is necessary as both ammonia and ammonium are toxic compounds in high enough concentrations, assimilation of ammonium also detoxifies it in the process. Throughout the plant ammonium assimilation is mainly done by the proteins of the glutamine synthetase - glutamate synthase system (GS/GOGAT) (Ohyama & Kumazawa, 1980). When ammonium is applied in high concentrations, increased concentrations of the GS/GOGAT enzymes are expected to detoxify the ammonium.

Diagram, venn diagram

Description automatically generated

Figure 2: GS/GOGAT pathway of both ammonia and ammonium. (Krupa, 2003)

In this process the free ammonium is converted into glutamine by glutamine synthase at the cost of ATP. Glutamate synthase then produces glutamate which can be used by most organisms for the production of amino acids, proteins and be used for the general metabolism.

## Nitrate uptake and regulation

Two nitrate transport systems have been uncovered thus far that take up nitrate from the soil and distribute it throughout the plant. The low-affinity transport system (LATS) facilitates nutrient uptake at high nitrate concentrations. LATS is mediated by *NRT1* gene family. Most of the nitrate transporters in the NTR1 family are proton-coupled transporters (Lin et al., 2008). The *NRT1* gene family consists of 53 genes in *Arabidopsis*. These are expressed in different tissues throughout the whole plant, which suggests that many of the genes in this family have a specific function (Tsay et al., 2007). An important aspect of the LATS is that the NRT1 transporter proteins have a diverse substrate specificity, which includes nitrate. This might make the *NRT1* gene family a more difficult target for molecular engineering as changing the expression levels could change the quantity of a large number of substrates which are transported into the plant cells.

The second nitrate transport system is the high-affinity transport system (HATS), which is has a role in the nitrate uptake under low nitrate conditions. The HATS is mediated by the *NRT2* gene family. NRT2.1 in *Arabidopsis* has an interaction with the NAR2 protein and together they form a major component of the HATS in *Arabidopsis* (Orsel et al., 2006). As shown by the *nrt2.1* *Arabidopsis* mutant losing up to 75% of high affinity nitrate uptake when compared to the wildtype (Filleur et al., 2001). In contrast to NRT1, the NRT2 proteins only transport nitrate (Forde, 2000). The NRT2 are proton coupled transporter proteins. In *Arabidopsis* 7 NRT2 transporters have been found thus far, with 4 of them showing nitrate deficiency related phenotypes when mutated (Wang et al., 2012). A strong correlation between the expression levels of the *NRT* genes and the nitrate uptake suggests that the regulation behind the transcription levels of these genes play a key role in the nitrate uptake. CHL1 or NRT1.1 in particular have been found to have a function in nitrate sensing and is linked to nitrate induction, nitrogen starvation upregulation and ammonium repression (Laugier et al., 2012). This could make it an interesting target when trying to increase a plants uptake efficiency of nitrate.

After the nitrate is taken up the nitrate is internally distributed into the plant tissues. The internal transportation is done by a variety of NRT family members which are expressed in specific tissues.

# Decreasing nitrogen over enrichment in crop farming

To decrease the amount of nitrogen fertilizer used in agriculture, an increase in NUE could prove extremely useful. To accomplish this, novel solutions are required to increase the NUpE of crops. Possible solutions are all based on reducing the input of fertilizer in the fields. There are however many ways to actually accomplish a fertilization reduction, including real time monitoring of the nitrogen content of plants, which might prove useful in decreasing the amount of over fertilization. Another solution could be the use of transgenic crops that have increased nitrogen uptake capabilities compared to their wildtype counterparts, also allowing the decrease of nitrogen input. These solutions will be discussed in this review.

Reducing the fertilizer application rate through modern agronomic practices without reducing yields should be a major objective of regulatory bodies to reduce the negative side effects of over enrichment. A case study in Australia showed that the recommended fertilizer dose can be reduced by up to 20%, while showing no loss of yield in cotton (Rochester et al., 2009). Although this might be applicable in many regions, it is dependent on the fertility of the soil, soil management and the yield potential (Janat, 2008).

Moreover, the optimal type of nitrogen fertilizer is also dependent on a variety of factors. If the nitrogen is applied in the form of ammonium for instance, the crops will acidify their rhizosphere which decreases the quantity of other cations the roots can take up. As some plants are more specialized in the uptake of either ammonium or nitrate which is dependent on their habitat, some crop species will also have a preference or specialization for the uptake of a certain type of nitrogen containing compound. Due to the enormous number of factors in play, it could prove difficult to successfully create strategies specifically for different soil types, in combination with crop species, different nitrogen fertilizer compounds and so on. Because the number of factors in play is so huge it might be more effective to focus on a smaller subset and to improve on those by as much as possible. One of these factors is the actual amount of nitrogen that the crops in the field have taken up from the soil.

## Measuring crop nitrogen content

Fertilizer is often applied in several different spraying sessions, which are spread over the growing season. The timings of these sessions are often predetermined and not influenced by accurate measurements of field nitrogen conditions. By accurately estimating the nitrogen content of the crops in the field, both spatial and temporal precision in applying fertilizers would become a possibility. Because the soil in an agricultural area is not homogenic, crops might have difficulty with the uptake of nitrogen in one subsection of the field whereas this could be different in another subsection of this same field. As this would lead to nitrogen deficiency stress, farmers might be inclined to increase the amount of fertilizer applied to the entire area, even though only a sub selection of the crops might even benefit from it. This directly leads to increased cost to farmers both monetarily and time wise. Moreover, over fertilization can also directly decrease the yield and quality of the crop.

Recent research showed the ability to estimate both the leaf nitrogen content and nitrogen use efficiency in wheat using sun induced chlorophyll fluorescence at both the leaf and canopy levels (Jia et al., 2021). Sun induced chlorophyll fluorescence (SIF) is a technique to surveil crop productivity, photosynthesis and physiology (Buschmann, 2007). The fluorescence is a byproduct after light is absorbed by the photosystem I and II protein complexes. The absorption of light reveals two emission peaks in the areas around both 690nm and 740nm of the light spectrum. As nitrogen has been proven to have a positive correlation with chlorophyll concentration (Evans, 1989), it is also expected that chlorophyll also has a positive correlation with the nitrogen content of leaves. SIF has been already used for the estimation of leaf nitrogen content at the leaf scale by Jia *et al* (2021). Recent developments show the possibility of SIF for leaf nitrogen content estimation at the canopy scale. The possibility of this technique in larger scale operations would prove extremely beneficial for real world application in crop farming.

In order to prove that this technique is viable at the canopy scale Jia et al. set out a field experiment where they compared the sun-induced chlorophyll fluorescence at the leaf and canopy scales. The hypothesis was that the leaf nitrogen content can be estimated at the canopy level by measuring the fluorescence with a spectrometer. The leaf nitrogen content is an important indicator for estimating the number of photosynthetic proteins that are present in the plant. They showed that the nitrogen content can be estimated in both an area based and mass-based leaf nitrogen content using this novel technique. They were also able to estimate the photosynthesis nitrogen use efficiency, based on the relationship between the sun induced fluorescence, photosynthesis data and the measurements of leaf nitrogen content.

Jia et al. determined that the leaf nitrogen content can indeed be estimated at both leaf and canopy scales. A caveat is however that the estimate of area-based leaf nitrogen content is more accurate than the estimation based on mass-based leaf nitrogen content. The results show that this technique allows the accurate estimation at the canopy level, which is a necessity in order for this technique to be useful in large scale agricultural use. Jia et al. also showed that sun induced fluorescence has the capability of correctly estimating the photosynthesis nitrogen use efficiency. These results can be used for further research into the change of nutritional status of crops over time. It can also be used to determine which crop variety is adapted to the local soil, as there will be differences in either leaf nitrogen content or photosynthetic nitrogen use efficiency between the different cultivars. Lastly it can also be used for more precise fertilizing practices by keeping track of the nitrogen content in the crops both over time and spatially.

Through both real time and nondestructive nitrogen concentration measurements famers will have a better idea of what the nitrogen enrichments levels of their fields are. This could thus prove to be an effective tool in the fight against the unnecessary overuse of fertilizer. Sadly, there could also be problems with the adaptation of this technology. The equipment that is necessary for the measurement of the fluorescence is still very much only focused on being used in science. This equipment would probably have to become cheaper for it to be a financially viable decision. It would probably take a long amount of time for farmers to make back their investment on the fluorescence reader through decreasing the applied fertilizer. Although it might be possible to share the equipment as it might only be necessary to measure the nitrogen content at some predetermined time points. This would decrease the cost and potentially increase the chance of real-world implementation. Furthermore, due to their price and complexity, farmers in less developed areas of the world might have difficulty accessing either the equipment itself or the knowledge of use. Time will tell if the measurement of nitrogen in crops will be a technique which is broadly adapted by the agricultural sector in the future.

# The use of transgenic crops to increase NUpE

Another possible solution to the overuse of fertilizer is the introduction of transgenic plant varieties. The number of crop characteristics that can be optimized through genetic modifications is very large. Compared to conventional breeding techniques, the use of genetic engineering is often both a cheaper and less time-consuming alternative. Even though the use of genetically modified organisms is not a common practice in for example the EU, different areas such as the USA have realized that the potential benefits could outweigh the hazards. It seems likely that the use of genetically modified crops will be globally accepted at some point, as the need for crop output will steadily climb due to the increasing world population. If an increase in nitrogen uptake efficiency can be gained through the use of genetic modification, this could be a major benefit to farmers in less developed areas of the world. If possible, this would be a one-time cost and time investment with long term benefits to both the farmers and their surrounding environments.

Transgenic technology has already been applied in plants by overexpression or knockout of genes. Genes can also be transferred within or across different species to either change preexisting gene expression levels or introduce new genes (Good et al., 2007).

As there are several parallel nitrogen uptake pathways for the different nitrogen containing compounds a wide variety of potential transgenic targets are available. Possibilities exist in using genes found in plant species or plant varieties that have specialized in living in soil containing low concentrations of nitrogen. When the genomes of these specialized plants are compared to the genomes of plants that do not have these adaptations, possible target genes could be uncovered which when introduced into different varieties might have a positive effect on reducing nitrogen deficiency stress through the increase of nitrogen uptake.

## The DP202216 mutant in maize

Recently researchers found that the transgenic *zmm28* maize line (Event DP202216) showed both increased N uptake and N utilization efficiencies (Fernandez et al., 2022). *Zmm28* was found in a transgenic discovery pipeline in which hundreds of maize transcription factors were fused to a gos2 promoter. The gos2 promoter provides a constitutive expression which is comparable to the Ubiquitin promoter in maize (Wu et al., 2019). This means that DP202216 is a has overexpression of the zmm28 gene.

Fernandez et al. validated that the introduction of a genetic transformation in a representative field environment could lead to real world benefits. *Zmm28* is one of the MADS-box genes which encode for transcription factors that are characterized by a highly conserved DNA binding domain, called the MADS-box, located at the N terminus of the protein.

DP202216 has been found to promote nitrogen uptake and nitrogen leaf allocation during the vegetative stage. In comparison to wildtype maize, DP202216 showed higher rates of nitrogen uptake during crop growth stages up to flowering. Importantly, this effect was found at both unfertilized and fertilized conditions. In later vegetative stages, this beneficial effect was lost however (Fernandez et al., 2022). The DP202216 hybrids were also found to have increased specific leaf nitrogen accumulation in the lower leaves of the canopy during stages before flowering. This increase was mostly apparent under the nitrogen fertilization treatment (Fernandez et al., 2022). These results indicate that the hybrid maize has a better response to the supply of nitrogen on nitrogen uptake and its storage in the leaves.

The DP202216 transgenic plants were also found to have a higher nitrogen allocation to the maize ears relative to the control. DP202216 also showed improved nitrogen remobilization from the stover to the maize grains. Under both unfertilized and fertilized conditions, DP202216 was found to have a higher proportion of nitrogen accumulated in the grains, compared to the WT control. This provides direct evidence that the hybrid DP202216 has increased nitrogen partitioning to the ear, which is achieved? through increased nitrogen remobilization (Fernandez et al., 2022).

Much remains unclear about the actual mechanism behind these increases as the DP202216 hybrid was created in a pipeline. It would however be probable that the internal transportation of nitrogen is increased from source to sink either through upregulation of these transporter genes or through the increased activation of their corresponding proteins. Likely targets are a subset of the NRT proteins that are active in the internal source to sink transport. The internally located AMT proteins could also be more active in these DP202216 hybrids, which would also lead to increased internal transport of nitrogen. This could be a part of the explanation the increase of nitrogen in the maize ears. The increase in uptake requires a different explanation however, the mildly constitutively expressed *zmm28* could also have elevated transcription of the AMT1 proteins that are responsible for the transport into the roots. The NRT proteins which are responsible for import of nitrogen might also be a target for increased transcription. Another possibility is that the increase in internal transport of nitrogen out of the roots leads to decreased concentrations of nitrogen in these root cells. This locally decreased nitrogen concentration could increase the amount of both passive and active nitrogen transport from the soil into the roots through a nitrogen stress signaling pathway. Several of the nitrate transporters also play roles in nitrate sensing (Wang et al., 2012). These transporters might thus play a role in the increased nitrogen uptake of the DP202216 hybrid, through the increased amount of signaling when larger amounts of nitrogen are transported away from the source tissues.

## The *NAC075* mutant in *Arabidopsis*

Besides using a transgenics pipeline to find potential beneficial molecular engineering targets in crops, it is also possible to target specific beneficial traits, such as for example: Plant root architecture. As the soil nitrate conditions are highly variable both in time and space in the environment (Undurraga et al., 2017), plants have evolved ways to respond to these variable conditions. In *Arabidopsis thaliana* nitrate availability influences the root architecture through a large signaling network. The *lonr1* mutant was recently identified to be less sensitive to low nitrate concentrations as the *NAC075* transcription factor is defective in this genotype (Xiao et al., 2022). Xiao et al. showed that *Arabidopsis thaliana* is able to regulate its root architecture through an adaptive mechanism which is modulated by nitrate deficiency. A key protein in this mechanism is the NAC075 transcription factor.

As nitrate is more abundant in deeper soil layers, deep plant root systems are generally more favorable for effective take up of nitrate (Lynch, 2013). Nitrate concentrations can either stimulate or inhibit root growth depending on the concentration. The *lonr1* mutant was found by isolating mutant lines that restored primary root growth in low nitrate concentrations to at least some extent. Both the *lonr1-1* and *lonr1-2­* showed lower sensitivity to the low nitrogen treatment compared to the wildtype. This difference in root growth gradually disappeared as the nitrate concentration increased, which directly indicates that the primary root growth of the *lonr-1* mutants is dependent on the nitrate concentration of the medium.

Xiao et al. also determined that the mutant seedlings showed normal nitrate levels in the shoots and roots similar to the wildtype in both normal and low nitrate conditions. The expression of the low affinity nitrate transporter NRT1.2 and of the high affinity nitrate transporter NRT2.1 did not change in the *lonr1* mutants under low nitrate conditions. This supports the idea that the *lonr1* mutants are specifically resistant to changes in root architecture under low nitrate conditions while showing nitrate uptake and accumulation levels that are similar to the wildtype.

Through the use of whole genome sequence analysis, it became clear that a mutation in the At4g29230 gene was responsible for the *lonr-1* phenotypes. The At4g29230 gene encodes for the NAC transcription factor *NAC075*. Analysis of the *NAC075* promoter showed that the gene is expressed in a variety of tissues (Kan et al., 2021). Using GFP fusions xiao et al. showed that NAC075 moved from the stele to the endodermal layer in the roots. This intercellular movement from the stele to the endodermal layer was shown to be inhibited under low nitrate concentrations. A non-movable *NAC075-3xGFP* mutant was created which showed the necessity of NAC075 intercellular movement for its role in the primary root adaptation. The data showed that NAC075 intercellular movement is required for the adaptation of primary root growth in response to low nitrate concentrations.

Xiao et al. showed that the ability of plant root growth adaptability to low nitrate concentrations is attained through a regulatory mechanism which is mediated by the CIPK1-NAC075 complex. CIPK1 is an interaction partner of NAC075, in this interaction the CIPK1 kinase phosphorylates the NAC075 protein. Under high nitrate conditions the CIPK1 is not activated and is not able to phosphorylate NAC075. This allows the NAC075 to freely move from the stele to the endodermal cells. Under low nitrate conditions however the opposite is true as the CIPK1 protein becomes activated and is able to phosphorylate the NAC075 which restricts the intercellular movement of the protein. This enables NAC075 to increase transcription of the WRKY53 gene, which has a direct stimulating effect on the root plasticity in response to nitrate deficiency.

As nutrients are not distributed homogeneously throughout the soil, plants have to constantly adapt their root architecture to optimize their nutrient uptake. The research done by Xiao et al. provides deep insight into the mechanisms in which the CIPK1-NAC075 complex is involved. This knowledge can be used in the future to create genetically modified crops which have a better developed root systems when compared to wildtype crops. Maize lines that are capable of deeper rooting under low nitrate conditions have been shown to outcompete lines that did not have this capability (Zhan & Lynch, 2015). Additional research is required to identify additional components of the CIPK1-NAC075 pathway. As it stands now, it would be beneficial to find the effect of a *NAC075* mutant in actual crop species as the signaling could be different in actual crop species such as maize, wheat or rice. If successful, this would allow the creation of genetically modified crops which are better equipped to deal with lower nitrate concentrations, which in turn means less nitrogen input is required for these crops to flourish.

# Conclusion

When faced with the consequences of over applying nitrogen fertilizer, such as loss of local biodiversity and eutrophication of waterways, it seems clear that novel solutions are necessary. In this review several novel developments have been discussed which in the future could lead to the decrease of nitrogen over enrichment. By enabling farmers to measure the nitrogen content in their crops, a reduction of nitrogen input is likely as over fertilization would also occur less often. Even though the technology might still need extra development time, especially in order to be useful for the agricultural market, there is certainly potential. The upside of this technique is that is can be easily combined with other solutions, such as the other solutions that have also been discussed in this review. The second solution discussed is the use of transgenic crops. Two distinct genetically modified crops have been discussed above. While both of them showed an increase in nitrogen uptake, much remains unclear about what the molecular mechanism behind the increase in nitrogen uptake in the DP202216 hybrid actually is. While speculation is possible, more research might be required to find out whether the hybrid has an actually viable use in agriculture in the future. For the *NAC075* mutant in *Arabidopsis* much more is clear about the mechanisms which induce the root growth. The limiting factor for this however is that the study was done in *Arabidopsis,* which could mean that the results are not directly translatable to important crop species. Due to the large number of possible targets in genetical engineering many more target genes are available. A combination of several beneficial traits might be the optimal way to improve nitrogen uptake efficiency. A combination of several techniques seems to be the clear solution to reduce the over enrichment by as much as possible, as many of the possible solutions are not mutually exclusive.

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