



MARIEME DRAME

**Impacts of Urease Inhibitors on Nitrogen Assimilation in Wheat
and on Reducing Nitrogen Losses**

School of Water, Energy & Environment
PhD Environment and Agrifood

PhD
Academic Year: 2019 - 2024

Supervisor: Professor Guy J.D. Kirk, Dr Alison Carswell, Professor Tom
Misselbrook
Associate Supervisor: Dr Mark Pawlett, Dr Martin Jemo
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This thesis is submitted in partial fulfilment of the requirements for the degree of PhD

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Abstract

A major global challenge for the 21st century is to ensure food security and sustainable development while limiting the adverse impact of agricultural reactive nitrogen (Nr) pollution and global warming greenhouse gas emissions (GHG). Climate change mitigation and adaptation strategies, with varying effectiveness, have been implemented across different regions in this perspective. The use of enhanced efficiency N fertilisers (EEF) in agriculture is a potential management strategy towards this aim, with documented benefits but with existing knowledge gaps. In this thesis, the efficacy of EEF, particularly urease inhibitors (UI), was determined under warmer, dryer climatic conditions as are expected to occur more frequently under climate change scenarios. Additionally, the potential of UI to improve nitrogen use efficiency (NUE) and enhance the tolerance of wheat varieties to drought stress at different N rates was evaluated, as well as the influence of UI on plant N assimilation and potential contribution to nitrous oxide (N₂O) emissions. The effects of high soil temperature (>25°C) and low soil moisture (<40% water filled pore space; WFPS) on emissions of ammonia (NH₃) and N₂O following application of urea to soil was assessed, and the efficacy of UI in reducing N losses. The findings suggest that treatment of urea with UI effectively reduces NH₃ losses at temperatures reaching 35°C, although overall effectiveness decreases with increasing temperature and low soil moisture conditions. Nitrous oxide emission was not influenced by the presence of UI but was high at soil moistures <60% WFPS. Nitrous oxide emission was also measured from wheat plants grown in soil and in a hydroponic system under low (7 kg ha⁻¹) and high N (70 kg ha⁻¹) conditions. Plants emitted more N₂O under low N growth conditions when supplied with additional potassium nitrate compared with those supplied with urea treated with UI. However, insufficient evidence was obtained from the hydroponic experiment to confirm plant N₂O formation through nitrate assimilation pathways, other than its overall contribution to N₂O emissions. Furthermore, when applied to plants under drought stressed conditions, UI did not enhance wheat tolerance to drought or increase yield and NUE. Nitrogen assimilation was influence by UI, particularly leaf urea concentration which increased in the presence of UI. Similarly, application of urea included with UI at a high N rate (180 kg ha⁻¹) resulted in lower wheat biomass and yield. Varietal differences were also observed in plant N₂O emission, drought tolerance and NUE. Overall, the findings support the use of UI as a Nr

mitigation strategy under warm and dry conditions; however, for increased NUE and yield, appropriate fertiliser and crop management, specific to local conditions may be needed.

Keywords: Nitrogen fertiliser, Nitrogen metabolism, Urease inhibitor, Greenhouse gas emission, Ammonia emission, Nitrous oxide emission, Nitrogen use efficiency

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Rest In Peace!

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List of Abbreviations

AAT	Alanine Transaminase
ATP	Adenosine Triphosphate
C	Carbon
CEC	Cation Exchange Capacity
CIMMYT	International Maize and Wheat Improvement Center
CO	Carbon Monoxide
CO ₂	Carbon Dioxide
DCD	Dicyandiamide
DI	Deionised
DMPP	3,4-dimethyl-1H-pyrazole phosphate
DMPSA	4,5-dimethylpyrazole-1-yl-succinic acid
DS	Drought-stressed
DW	Dry Weight
EC	Electrical Conductivity
EDTA	Ethylenediaminetetraacetic Acid
EEF	Enhanced Efficiency Fertilisers
EU	European Union
FAD	Flavin Adenine Dinucleotide
FAO	Food and Agriculture Organisation.
FW	Fresh Weight
GDC	Glycine Decarboxylase
GDH	Glutamate Dehydrogenase
GHA	γ -glutamyl hydroxamate
GHG	Greenhouse Gas
Gln	Glutamine
Glu	Glutamate
GOGAT	Glutamate Synthase or Glutamine Oxoglutarate Aminotransferase
GS	Glutamine Synthetase
H ₂ O	Dihydrogen Monoxide
HCO ₃	Bicarbonate
IAEA	International Atomic Energy Agency
IPCC	Intergovernmental Panel on Climate Change

KNO ₃	Potassium Nitrate
MSO	Sodium Nitroprusside
N	Nitrogen
N ₂	Dinitrogen
N ₂ O	Nitrous Oxide
N ₂ OR	Nitrous Oxide Reductase
NADH	Nicotinamide Adenine Dinucleotide
Nar	Nitrate Reductase
NBPT	N-(n-butyl) thiophosphoric triamide
NH ₂ OH	Hydroxylamine
NH ₃	Ammonia
NH ₄	Ammonium
NI	Nitrification Inhibitors
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NiR	Nitrite Reductase
NO	Nitric Oxide
NO ₃	Nitrate
Nor	Nitric Oxide Reductase
NO _x	Nitrogen Oxides
NPK	Nitrogen Phosphate Potassium
NPPT	N-propyl- thiosphosphoric- triamide
Nr	Reactive Nitrogen
NR	Nitrate Reductase
NUE	Nitrogen Use Efficiency
O ₂	Oxygen
OCP	Office Chérifien des Phosphates
REML	Linear Mixed Effect Model
RT	Room Temperature
SAFA	Sustainable Agriculture for Africa
SNP	Sodium Nitroprusside
TCA	Tricarboxylic Acid
TOC	Total Organic Carbon
TN	Total Nitrogen

UI	Urease Inhibitors
UK	United Kingdom
WEF	World Economic Forum
WFPS	Water Filled Pore Space
WHC	Water Holding Capacity
WW	Well-watered
2-NPT	N-(2-nitrophenyl) phosphoric triamide
2-OG	2-oxoglutarate

1 Improving Crop Nitrogen Use Efficiency in a Changing Climate

1.1 Introduction

Increasing global population augments the need for greater food production to ensure global food security (Govindasamy et al., 2023). This requires extending agricultural land or producing more food through intensified agriculture (Fitton et al., 2019), while also providing sustainable economic growth and reducing poverty (International Atomic Energy, 2024). Climate change will make this task more difficult and remains a significant threat to food security and sustainable development for many populations worldwide (Ratnayake et al., 2023). Extreme weather and climate events (heatwaves, heavy precipitation, droughts, and tropical cyclones) have increased vulnerability to food insecurity (IPCC, 2023). Crop production is particularly susceptible to changing trends in global temperature because elevated temperatures have substantial adverse effects on crops (Abbass et al., 2022). Zhao et al. (2017) reported yield reduction of major crops including wheat, rice, maize, and soybean, by 6.0, 3.2, 7.4 and 3.1%, respectively, with an increase of 1°C in global mean temperature.

Human activities have contributed significantly to global warming, predominantly through the production of greenhouse gas (GHG) emissions, which continue to increase, contributing to a warming of 1.0 to 2.0°C between 2010 and 2019 (IPCC, 2023). Agricultural, forestry and other land use activities accounted for 22% of the global GHG emitted in 2019 (IPCC, 2023). Emissions from agriculture principally arise from the substantial use of inorganic fertilisers and manure to produce food (Bell et al., 2015). Providing plants with nitrogen (N) and other essential macro and micronutrients for plant development has increased yield and economic growth over the past century (Javed et al., 2022). After water, nitrogen is a fundamental requirement for crop growth (Govindasamy et al., 2023). Nitrogenous fertilisers account for over 90% of the total manufactured fertilisers globally (Tyagi et al., 2022). In low-income and advanced countries, excessive N application is a common practice by farmers to guarantee high crop yields, but only 30-50% of the applied N is assimilated by crops (Liang et al., 2018). The remainder, or excess N, is lost through different pathways to the surrounding environment where it contributes to environmental pollution (soil, air, water bodies), and climate change (Govindasamy et al., 2023; Javed et al., 2022; Tyagi et al., 2022). Nitrogen can also be

lost directly from crop plants in the form of ammonia (NH_3 , Schjoerring and Mattsson, 2001) and possibly as nitrous oxide (N_2O , Lenhart et al., 2019), highlighting that plant processes may also significantly affect the N budgets in agricultural systems (Kumagai et al., 2011; Schjoerring and Mattsson, 2001; Sommer et al., 2004).

Crop production needs to increase by 60% to meet projected demands by 2050 (International Atomic Energy, 2024) which may require increased use of inorganic fertilisers to supply sufficient plant nutrients and sustain food production. Therefore, efforts should be made for efficient nutrient use, particularly N, for sustained soil health and to reduce the environmental footprint of agriculture (Hakeem et al., 2016). In this context, various countries and international bodies have established environmental targets and legislation to reduce nitrogen losses. These targets primarily focus on limiting N emissions to air and reducing N leaching and runoff into water bodies. Among others, the European Union Nitrates Directive aims to develop action programs to control nitrate losses and water pollution from the use of fertilisers in agriculture and identify Nitrate Vulnerable Zones (European Commission, 2024). The UK Clean Air Act targets the reduction of major transboundary air pollutants emissions including NH_3 and fine particulate matter ($\text{PM}_{2.5}$) from major sources (DEFRA, 2019) caused by transboundary air pollution. The UK Net Zero Strategy intends to reduce GHG emissions to 100% by 2050 relative to 1990 levels and mitigate and adapt to the impacts of climate change (DEFRA, 2023). Consequently, optimising nitrogen use efficiency (NUE) between 50 and 90% (EU Nitrogen Expert Panel, 2015) is a critical goal in crop enhancement programs and agronomic management frameworks (Govindasamy et al., 2023). Nitrogen use efficiency has multiple definitions (Dobermann, 2007) relating to how much N is supplied to the crop and how much is harvested in the grain/crop yield. Enhancing NUE through appropriate agronomic management practices and technologies would decrease the need for heavy N application and minimise the negative environmental impacts (Govindasamy et al., 2023).

Enhanced efficiency fertilisers (EEF) delay the release of N to plants by blocking the active site of enzymes, which process N for one form to another, or release N at a slower rate (slow-release or controlled-release fertilisers) for enhanced plant uptake, which can minimise losses and improve crop productivity per unit of fertiliser applied (Dimkpa et al., 2020; Halvorson et al., 2014). A variety of EEF are available and are generally

classified depending on their mode of operation on the N cycle, such as urease and nitrification inhibitors, and the technologies used in their development, such as slow and controlled-release fertilisers (Abalos et al., 2014; Dimkpa et al., 2020; Trenkel, 2010).

Furthermore, many studies have shown that genetic variation in wheat can affect NUE and that using the best-adapted genotype has the potential to improve efficiency in how crops acquire and use soil N or fertiliser N (Belete et al., 2018). Different varieties of crops which are responsive to fertilisers have historically been introduced to sustain food demand, predominantly in low-income countries like India, which contributed to the Green Revolution in agriculture (Tyagi et al., 2022).

However, changing climate and extreme weather events will substantially influence nutrient cycling processes in soil and, potentially, the effectiveness of EEF and the ability of plants to acquire nutrition from the soil and applied fertilisers. Currently-used EEF have been reported to be influenced by various factors, such as variations in soil properties and environmental and climatic conditions (Abalos et al., 2014). Therefore, future climate scenarios may limit their potential to mitigate N losses.

1.2 Nitrogen cycle processes and influencing factors

1.2.1 Soil nitrogen cycle

Nitrogen is predominantly present in the atmosphere in its unreactive form as dinitrogen (N_2) (Hofman and Cleemput, 2004; Jensen and Hauggaard-Nielsen, 2003) comprising 79% of atmospheric gases (Shafreen et al., 2021). In soil, the inorganic forms of N most accessible to plants are limited (Hofman and Cleemput, 2004) as more than 95% of the soil N is present as organic forms (Walworth, 2013), including proteins, amino acids, nucleic acids, chitin, in living organisms (plants and animals) and complex N compounds including humus (Butterbach-Bahl et al., 2013; Kakraliya et al., 2018). The inorganic forms of N include ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), and nitrogen oxides (NO_x), and N_2 (Havlin et al., 2014; Shafreen et al., 2021). Ammonium and NO_3^- are the inorganic N forms in soil most readily taken up by plants, deriving from organic matter decomposition or the application of fertilisers in agricultural ecosystems (Havlin et al., 2014), although other N compounds such as amino acids, peptides and urea are also readily available for plant uptake where they are accessible (Tegeder and Rentsch, 2010; Witte, 2011).

In agricultural systems, the N cycle (Figure 1-1) is complex due to imbalances between N inputs and outputs, disturbing the equilibrium found in natural ecosystems (Hofman and Cleemput, 2004). The soil N cycle includes microbiological processes such as N fixation, mineralisation, immobilisation, nitrification and denitrification, and physical processes such as volatilisation and leaching (Hofman and Cleemput, 2004; Kakraliya et al., 2018). These N cycle processes (Table 1-1) are regulated by different environmental factors, soil physicochemical characteristics, and their interactions (Schlüter et al., 2018; Signor and Cerri, 2013).

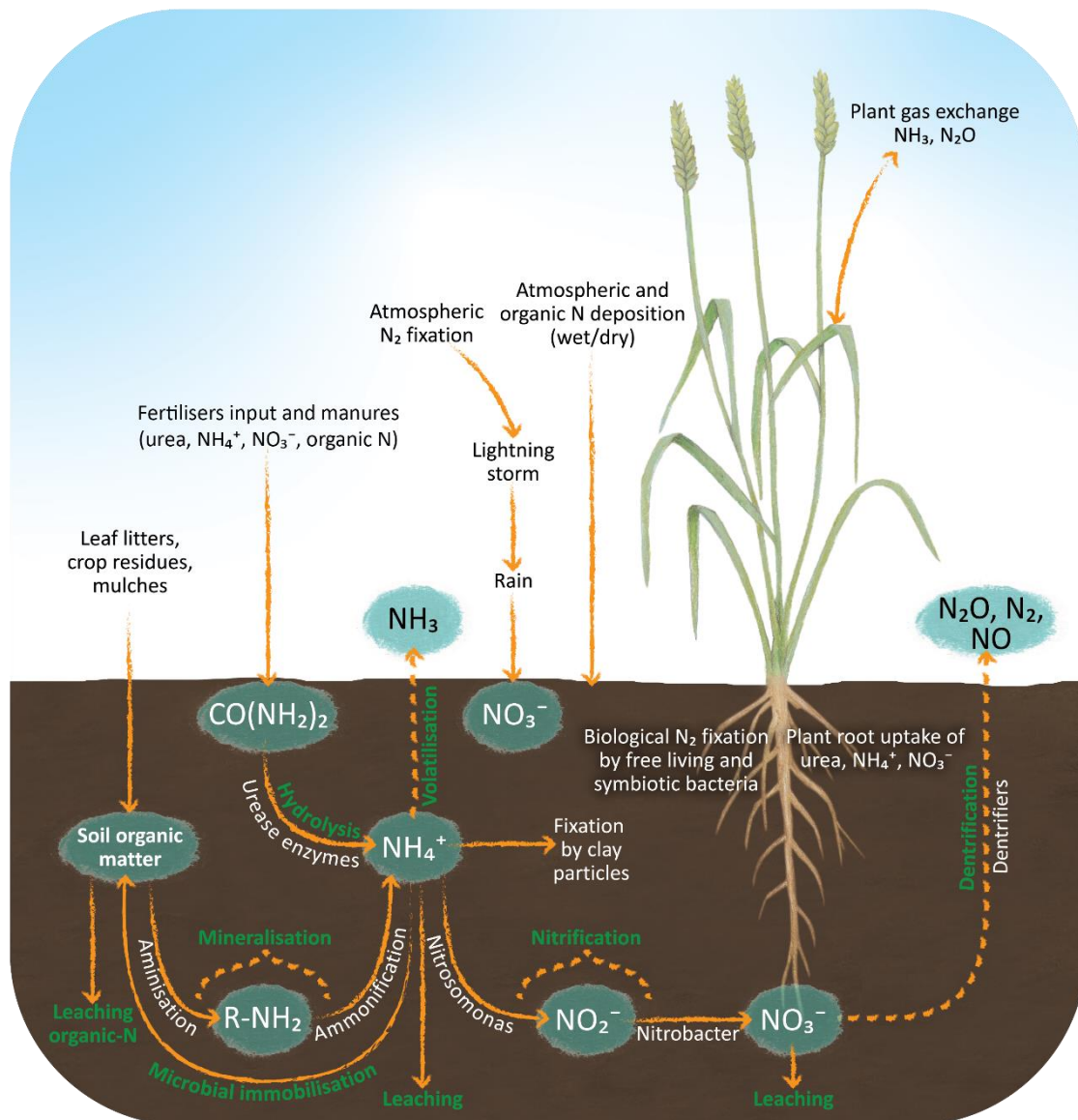


Figure 1-1 : The soil N cycle processes

Table 1-1 : Nitrogen cycle processes

Process	Determinant	References
Nitrogen fixation	<p>N₂ fixation by the combined reaction of lightning and oxygen (O₂) from the air gives rise to NO_x (nitric oxide followed by nitrogen dioxide). The electric discharge from the lightning strikes catalyses the first oxidation reaction. With rainfall, the NO₂ reacts with rainwater to form nitrous acid (HNO₂) and nitric acid (HNO₃), which also react with alkaline radicals from soil to form water-soluble NO₃⁻ and NO₂⁻.</p> <p>Industrial N₂ fixation with the Haber-Bosch process involves the reaction of N₂ with hydrogen from water at high temperatures (400-500°C) and pressure (15-25 MPa). This reaction produces NH₃, which can be further transformed into different N fertiliser forms.</p> <p>Biological fixation of N₂ by free-living (autotrophic or heterotrophic bacteria) and symbiotic (with legumes and algae) N-fixing organisms. This N fixation is mainly driven by nitrogenase enzymes, which catalyse the production of NH₃ and N-containing organic compounds (e.g., amino acids, nucleic acids, protein).</p>	(Bernhard, 2010 ; Havlin et al., 2014 ; Hofman and Cleemput, 2004; Jensen and Hauggaard-Nielsen, 2003; Kakraliya et al., 2018)
Mineralisation	<p>Mineralisation is favoured when the supplied or existing organic matter has a carbon (C)-to-nitrogen (C:N) ratio below 20:1. Mineralisation involves two reactions:</p> <p>Aminisation through enzymatic decomposition of proteinaceous material and proteins from crop residues and soil organic matter by aerobic and anaerobic bacteria, fungi and actinomycetes into amino acid amines and urea.</p> <p>Ammonification through the transformation of amines and amino acids by bacteria (aerobic and anaerobic), fungi and actinomycetes to produce inorganic N, particularly NH₄⁺ and/or NH₃, depending on soil pH.</p>	(Abera et al., 2012 ; Galloway, 2003 ; Havlin et al., 2014; Kakraliya et al., 2018; Persson et al., 2000)
Immobilisation	<p>Immobilisation is the assimilation of inorganic N (NH₃/NH₄⁺, NO₂⁻, NO₃⁻) by microorganisms and mycorrhizal fungi to produce protein for their growth. Immobilisation is induced when the supplied or existing organic matter has a C:N ratio above 30:1.</p>	(Abera et al., 2012; Hofman and Cleemput, 2004; Persson et al., 2000;

		Walworth, 2013)
Nitrification	<p>Nitrification is a two-step process: The oxidation of NH_4^+ or NH_3 via hydroxylamine (NH_2OH) by ammonia-oxidizing (i.e., Nitrosomonas species) to produce NO_2^- Oxidation of NO_2^- into NO_3^- by nitrite-oxidizing (i.e., Nitrobacter species) autotrophic bacteria. Small amounts of N_2O and NO are released during nitrification, potentially through a reductive pathway defined as nitrifier denitrification. Heterotrophs can perform nitrification at a lower rate than autotrophs; some fungi and actinomycetes can also transform NO_2^- to NO_3^-. Archaea can oxidize NH_4^+ to NO_2^-.</p>	(Bernhard, 2010; Butterbach-Bahl et al., 2013; Galloway, 2003; Hofman and Cleemput, 2004; Kakraliya et al., 2018; Ward et al., 2019)
Denitrification	<p>There are two types of denitrification: Biochemical denitrification which is the reduction of NO_3^- and NO_2^- under anaerobic conditions by anaerobic bacteria into NO, N_2O and N_2. This is a heterotrophic process that requires an organic substrate. Chemodenitrification which is the reduction of NO_3^- and NO_2^- under anaerobic conditions but catalysed by primary minerals obtained under reducing conditions (e.g., Fe^{2+}) into NO, N_2O and N_2. This non-biological process is dominant in acid conditions and takes place predominantly in the subsoil.</p>	(Bernhard, 2010; Galloway, 2003; Havlin et al., 2014; Hofman and Cleemput, 2004; Kakraliya et al., 2018; Signor and Cerri, 2013)
Nitrate leaching	Nitrate leaching is the loss of NO_3^- from the plant root zone into receiving waterbodies. Leaching of NH_4^+ and dissolved organic N can also occur.	(Hofman and Cleemput, 2004; Persson et al., 2000)
Ammonia volatilisation	Ammonia volatilisation is generated from NH_4^+ during mineralisation of organic material and hydrolysis of inorganic fertiliser (e.g., anhydrous NH_3 , NH_4^+ or urea), which is a reversible reaction dependant on pH.	(Hofman and Cleemput, 2004; Kakraliya et al., 2018; Rochette et al., 2009)

1.2.1.1 Temperature and moisture effects on nitrogen cycling processes

Increased soil temperature accelerates soil microbial activity and respiration, increasing the rate of N transformation processes (Signor and Cerri, 2013). Nitrification has long been known to generally follow a bell-shaped temperature response curve with an

optimum of 30-35°C (Sahrawat, 2008). However, nitrification is climate-dependent because tropical soils appear to have a higher optimum temperature range for nitrification than soils in temperate regions (Sahrawat, 2008). This is because nitrifier species in temperate areas have lower temperature optima and are, therefore, more sensitive to warm conditions than species from hotter regions (Hofman and Cleemput, 2004). Ammonia gas is formed during the mineralisation of soil organic N, surface applied N or incorporated N (Hofman and Cleemput, 2004). The rate of this process also increases with temperature, with the increase in microbial activity leading to increased volatilisation of NH_3 (Shafreen et al., 2021). Nonetheless, even at near-freezing temperatures, NH_3 volatilisation can still be an important N loss pathway (Dari et al., 2019). Denitrification of NO_3^- is also sensitive to temperature and shows high spatial and temporal variation related to so-called hotspots and hot moments (Butterbach-Bahl et al., 2013; Schlüter et al., 2018). An increase in temperature has been reported to increase the $\text{N}_2/\text{N}_2\text{O}$ emission ratio, reducing N_2O emissions relative to N_2 (Maag and Vinther, 1996). Nevertheless, N_2O can be emitted at temperatures close to 0°C since microbial activity can still occur under these conditions. The freeze/thaw phenomenon also induces pulses of N_2O emissions, because of enhanced microbial activity during soil thawing in the spring on topsoil (Wolf et al., 2010).

Nitrification is a biological process occurring in well-aerated soils (House and House, 2016). Diffusion of O_2 molecules into the soil is often restricted at higher soil moisture above field capacity, as most of the soil pore spaces are filled with water (Subbarao et al., 2006), which affects nitrifier activity (House and House, 2016; Singh, 2007). Therefore, nitrification is restrained under low O_2 and high soil water content. Nitrifiers are autotrophs, i.e., they rely mostly on atmospheric CO_2 for their C requirements (Subbarao et al., 2006). Consequently, reduced concentration of CO_2 in the soil limits nitrification. In contrast, denitrification is an anaerobic biological process where the microorganisms use NO_3^- and NO_2^- as O_2 sources (Havlin et al., 2014). Thus, soil aeration determines the prevalence of aerobic or anaerobic microbial activity in soil (Hofman and Cleemput, 2004). Under saturation conditions of >70% water-filled pore space (WFPS), N_2O emission is predominantly sourced from the denitrification process. However, at $\leq 60\%$ WFPS nitrification is the main source of N_2O emission (Signor and Cerri, 2013) principally because the production of the proteome (e.g., Nar, Nir, Nor and N_2OR)

inducing microbial denitrification is stimulated under O₂ depleted conditions. In contrast, its synthesis is inhibited at high O₂ conditions (Bakken et al., 2012).

Volatilisation of NH₃ can be substantial under moist conditions, particularly from surface-applied N fertiliser such as urea, because of enhanced urea hydrolysis rate, reduced urea movement into the soil and sustained contact of urea granules with the damp soil surface (Dari et al., 2019). However, volatilisation of urea decreases when the soil reaches saturation (Jones et al., 2013) because gas diffusion through the soil is reduced. In addition, rain events and irrigation after surface application of N fertiliser minimise NH₃ volatilisation by transferring the fertiliser into the soil (Dari et al., 2019; Jones et al., 2013). Windy conditions and drying soil also favour NH₃ volatilisation from surface-applied urea (Dari et al., 2019; Mikkelsen, 2009).

Nitrate is a highly soluble ion loosely held in soil because most agricultural soils have a low anion exchange capacity (Havlin et al., 2014). Nitrate moves through the soil profile by diffusion and with the mass flow of water. Therefore, increasing water movement (e.g., under heavy rainfall and irrigation) and NO₃⁻ availability can promote NO₃⁻ leaching (Hofman and Cleemput, 2004). In addition, N can be lost via hydrologic pathways as other forms of dissolved N and particulate N, such as organic N and NH₄⁺, which can be adsorbed on soil particles (fine clay particles; Hofman and Cleemput, 2004).

1.2.1.2 Soil factors affecting nitrogen cycling processes

Nitrification is also affected by soil physical (texture and structure) and chemical (pH, electrical conductivity (EC), C:N ratio, cation exchange capacity (CEC), and organic matter content) properties (Kakraliya et al., 2018; Subbarao et al., 2006). The presence of NH₄⁺ ions in the soil is the initial requirement for nitrification; thus, when NH₄⁺ availability is restricted, nitrification is low (Havlin et al., 2014). Ammonium availability in soil is influenced by factors constraining organic matter mineralisation such as the C:N ratio, but also by absorption of NH₄⁺ into soil particles (Hofman and Cleemput, 2004). Since nitrifiers grow on the surface of soil particles close to NH₄⁺ ion attachment sites, the greater the absorption, the higher the nitrification rate (Subbarao et al., 2006), making soil CEC an important factor for the nitrification process. Soil pH is the major factor regulating soil nitrification, which occurs at a soil pH ranging between 5.5 to 10.0, with

the optimum pH of 8.5 (Sahrawat, 2008). The nitrification of NH_4^+ releases protons, which acidify the soil, so the natural buffering capacity of the soil influences the nitrification rate (Subbarao et al., 2006); soils with a low buffering capacity will become acidified more rapidly (Jones et al., 2013; Subbarao et al., 2006) and a low soil pH reduces the rate of nitrification (De Boer and Kowalchuk, 2001; Subbarao et al., 2006).

Ammonia gas is generated in the soil-plant system during nitrification of NH_4^+ (Hofman and Cleemput, 2004; Kakraliya et al., 2018) and its volatilisation is particularly high from certain surface-applied synthetic N fertilisers (e.g., anhydrous NH_3 , urea and NH_4^+) and livestock manure (Havlin et al., 2014). Potential for NH_3 volatilisation is greatest in alkaline soils ($\text{pH} > 7.5$), where the rate of NH_4^+ converted into NH_3 is greatly increased (Havlin et al., 2014). After urea fertiliser is applied, soil pH surrounding urea granules temporarily rises during urea hydrolysis (to a $\text{pH} > 8$), inducing NH_3 loss even in acid soils (Mikkelsen, 2009). Soil buffering capacity is related to CEC, clay content, soil organic material or a combination of these, which therefore influence the NH_3 to NH_4^+ ratio and hence volatilisation (Dari et al., 2019).

Organic substrates sustain heterotrophic denitrification (Hofman and Cleemput, 2004) by increasing anoxia in micro-sites (Schlüter et al., 2018). The size of soil aggregates can affect N_2O emission, with small-sized aggregates rapidly reaching anoxic conditions favourable to denitrification, compared to larger aggregates which take longer (Schlüter et al., 2018). Soil pH also influences denitrification by impacting denitrifier communities and the extent to which full denitrification (through to an end-product of only N_2) occurs (Bergaust et al., 2011; Schlüter et al., 2018). A negative correlation between pH and the proportion of denitrification end-product as N_2O has been reported in agroecosystems (Bakken et al., 2012) due to low pH inhibiting the production of N_2O reductase enzyme (N_2OR ; Bakken et al., 2012). Additionally, soil nutrient deficiency in copper and zinc has been reported to influence some denitrifiers, shifting the ratio towards N_2O (Gaimster et al., 2018; Sullivan et al., 2013). Nitrogen fertilisation with NO_3^- and NO_2^- from synthetic or organic fertiliser applications can increase soil N_2O production.

1.2.1.3 Effects of extreme weather events on N cycling

Extreme climatic events such as floods, droughts and heatwaves have been shown to significantly influence soil N cycling with profound implications on plant and microorganism biodiversity, agroecosystem productivity, and the balance between N sources and sinks (Barrat et al., 2021; Dodd et al., 2023; Qu et al., 2023). Flooding and drought alter substrate diffusivity and soil aeration, which impact plant growth and microbial community at the low and high extremes particularly (Dodd et al., 2023; Qu et al., 2023). Nevertheless, flooding and drought may influence natural soil processes differently and determine ecosystems' resistance and resilience to flooding and drought. Flooding, for instance, can lead to anoxic soil conditions which promote denitrification. Dodd et al. (2023) observed significantly lower soil redox potential (-92 mV) during prolonged flooding relative to drought or normal conditions ($\geq +200$ mV), which was associated with soil anaerobic conditions. Consequently, this could result in an increase in soil-dissolved organic C and phosphorus (P), NH_4^+ , and NO_3^- due to inhibited nitrification during flooding, and enhanced mineralisation of organic matter accumulated from decomposed plant material post-flooding (Wright et al., 2015; Qu et al., 2023). Under increased summer temperatures post-flood, the change in plant and microbial community structure could accentuate soil processes resulting in a pulse of C and N in soil (Dodd et al., 2023; Kirwan and Blum, 2011; Sánchez-Rodríguez et al., 2019). These conditions caused by prolonged flood stress can result in large GHG emissions (including CO_2 , CH_4 and N_2O) during soil recovery and when fertiliser is applied (Dodd et al., 2023; Qu et al., 2023). Flooding was reported to produce the highest GHG emission (as global warming potential; GWP but excluding CO_2) than that caused by N fertilisation, particularly when followed by a drought event (Dodd et al., 2023; Hou et al., 2000).

Conversely, drought conditions reduce microbial activity and respiration, slowing down processes like nitrification and mineralisation and favouring organic substrate accumulation with the length and intensity of drought (Borken & Matzner, 2009; Qu et al., 2023). Substrate (e.g., NH_4^+ and NO_3^-) accumulation under drought could be induced by the limitation of plant growth and reduced leaching potential of ions (Dodd et al., 2023). Substrates could also derive from drought-induced accumulation of dead microbial and plant cells, lysis of living microbial cells, microbial release of solutes after rewetting, and enhanced mineralisation of C and N after rewetting of soils (Borken & Matzner,

2009). The physical disruption of soil aggregates also contributes to the flush of mineralised C and N and subsequent N₂O emission after rewetting of dry soil (Borken & Matzner, 2009; Guo et al., 2014). The meta-analysis from Barrat et al. (2022) revealed that the duration and severity of drought can influence the soils potential to generate N₂O during recovery (i.e., rewetting of dry soil) with the pulse of emission generally greater at higher WFPS conditions (although the relationship may be non-linear). For instance, the size of a N₂O hot moment was found to be higher with a relatively larger change in water potential between the drought period and rewetting of dry soil (e.g., from 40 to 90% WFPS; Barrat et al., 2022). Hot moments' N₂O production contributes significantly to annual fluxes (Barrat et al., 2022; Song et al., 2022).

Heatwaves, exacerbate N cycle processes by accelerating volatilisation of NH₃, N₂O emission and enhancing the decomposition of soil organic matter, leading to microbial mineralisation, nitrification and denitrification in terrestrial ecosystems (Dai et al., 2020). The findings of Xu et al. (2017) suggest that soil microorganism response to extreme heat varies depending on microbial communities and the available N source, which also impacts microbial growth and gaseous N losses. Overall, extreme weather events create a more variable and less predictable N cycle, challenging the stability of ecosystems and agricultural productivity and potentially leading to increased GHG emissions.

1.2.2 Plant nitrogen cycle

In agricultural systems, the N is taken up by plants for the synthesis of proteins, amino acids, nucleic acids, and other plant N-components, as NH₄⁺ or NO₃⁻ (Magray et al., 2022; O'Brien et al., 2016; Ohyama, 2010) and other organic N forms such as urea and amino acids. Further, plants can absorb N via their leaves, from foliar N applications or N deposition, or from fixation by nitrogenase and absorption of amino acids (Magray et al., 2022; Ohyama, 2010). Plant preference for NH₄⁺ or NO₃⁻ relates to plant species, soil and environmental conditions (Guo et al., 2022; Krapp, 2015; Ohyama, 2010). For instance, NO₃⁻ predominates in well-aerated soils, whereas the opposite is true in water-logged paddy soil (O'Brien et al., 2016; Ohyama, 2010) and acidic soil (Krapp, 2015). Nevertheless, NH₄⁺ is the central component in plant N assimilation into other plant-N components (Figure 1-2) and NO₃⁻ is reduced into NH₄⁺ after absorption (Ohyama, 2010).

Ammonium can also be provided from photorespiration and N recycling (protein degradation) pathways (Krapp, 2015; Ohyama, 2010).

1.2.2.1 Ammonium assimilation

Glutamine synthetase (GS) and glutamate synthase (GOGAT) are the main enzymes involved in NH_4^+ assimilation in plants (Krapp, 2015; Ohyama, 2010). Two GS isozymes have been identified, one in the cytosol (GS1) associated with the recycling of NH_4^+ at specific growth stages (e.g., senescence) and in glutamine (Gln) synthesis for transport into the phloem sap (Bernard and Habash, 2009; Masclaux-Daubresse et al., 2010) and the second in the chloroplast or plastid (GS2), which is involved in the primary assimilation of NH_4^+ from NO_3^- reduction and from photorespiration and the recycling of amino acids (Masclaux-Daubresse et al., 2010; Ohyama, 2010). Once in leaf cells, the absorbed or recycled (from amino acid catabolism, phenylpropanoid pathway, photorespiration, rhizobia NH_4^+ fixation) NH_4^+ is primarily assimilated into Gln from glutamate (Glu) and adenosine triphosphate (ATP) through GS enzyme reaction (Kishorekumar et al., 2020). Then GOGAT transfers the amide glutamine group into 2-oxoglutarate (2-OG), which forms two molecules of Glu (Kishorekumar et al., 2020; Ohyama, 2010; Figure 1-2). Higher plants have two types of GOGAT including Fd-GOGAT which requires reduced ferredoxin (Fd) as a reductant present in leaf chloroplasts and NADH-GOGAT requiring nicotinamide adenine dinucleotide (NADH) found in non-photosynthetic tissues (e.g., roots, stems, etiolated leaf tissues, etc.; Krapp, 2015; Masclaux-Daubresse et al., 2010). Glutamine is also the precursor of various amino acids in chlorophylls, synthesised in the tricarboxylic acid (TCA) cycle (Kishorekumar et al., 2020). During the NH_4^+ assimilation process, plants can release NH_3 (Figure 1-2) when the atmosphere concentration of NH_3 is lower than that in substomatal cavities within the leaves; a higher atmospheric NH_3 concentration results in absorption of NH_3 by plants (Schjoerring et al., 2000; Schjoerring and Mattsson, 2001).

1.2.2.2 Nitrate assimilation

Transport of NO_3^- from soil solution into plant cells requires energy because it is negatively charged, and the cell has a low electrical potential (Ohyama, 2010). Inside the plant, NO_3^- is first reduced into NO_2^- by nitrate reductase (NR) which requires NADH (Kishorekumar et al., 2020), present in the cytosol of plants roots or leaves (Kishorekumar

et al., 2020; Ohyama, 2010). Two types of NR can be found in higher plants; most plants only have the NADH-specific NR, while others have only nicotinamide adenine dinucleotide phosphate (NADPH)-bispecific NR (Kishorekumar et al., 2020; Ohyama, 2010). The NO_2^- is further reduced to NH_4^+ by nitrite reductase (NiR) in the plastid or chloroplast, which is then assimilated for amino acid biosynthesis via the GS/GOGAT cycle (O'Brien et al., 2016; Ohyama, 2010) and alanine transaminase (AAT) reactions (Kishorekumar et al., 2020). The surplus of NO_3^- is accumulated in the vacuoles for storage and osmoregulation (Kishorekumar et al., 2020). Direct leaf emissions of N_2O during NO_3^- assimilation have been reported (Lenhart et al., 2019; Smart and Bloom, 2001), although the mechanisms for this are not well known.

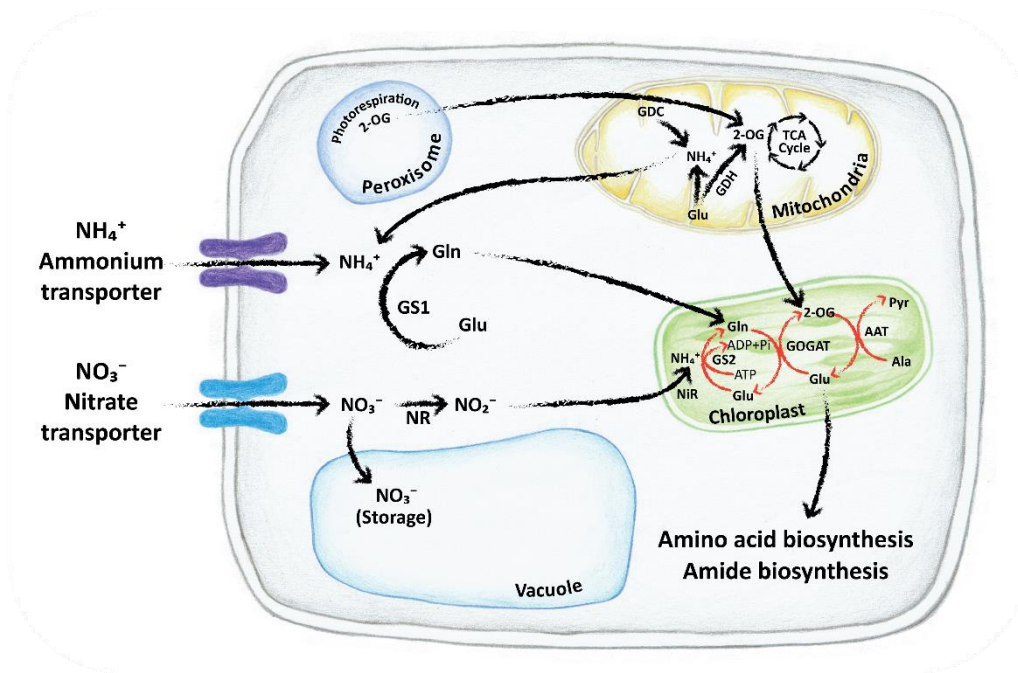


Figure 1-2: Nitrogen assimilation in plant leaf
Adapted from (Kishorekumar et al., 2020)

1.2.2.3 Factors influencing N uptake and assimilation in plants

Plant uptake of NH_4^+ and NO_3^- and subsequent assimilation can be influenced by several factors such as the soil NO_3^- and NH_4^+ content, soil pH, gas exchange, radiation, temperature, moisture and the presence of metals (Kishorekumar et al., 2020; Mokhele et al., 2012). Low levels of NO_3^- in soil and plants can reduce NR activity (Mokhele et al.,

2012); similarly, greater NH_4^+ concentration can limit NR activity (Kishorekumar et al., 2020). Glutamine synthetase is active even at low NH_4^+ concentrations, revealing its high affinity for NH_4^+ , with high NH_4^+ concentrations being toxic (Ohya, 2010). Soil solution pH can influence N availability and uptake by plants, but also the source of N as well as plant uptake of NH_4^+ or NO_3^- which can alter the rhizosphere pH (Shi et al., 2023) and the ionic and pH balance in plant cells (Feng et al., 2020). In winter wheat seedlings grown in neutral, acidic, and alkaline hydroponic solutions there was lower NR activity in roots and shoots at low pH, while GS activity was generally inhibited under extremely acidic and alkaline pH conditions (Shi et al., 2023).

Both NR and GS activity can be influenced by CO_2 concentration (Mokhele et al., 2012). High concentrations of atmospheric CO_2 or low concentrations of O_2 have been reported to reduce photorespiration, decreasing the level of NADH to drive NO_3^- reduction in C_3 plants and NO_2^- transport from the cytosol into the chloroplast (Bloom et al., 2010) with potential impacts on NH_4^+ assimilation. Additionally, plant development requires sunlight; however, high ultra-violet B-radiation can disrupt photosynthesis and the assimilation of NO_3^- and NH_4^+ , through inhibition of NR and GS/GOGAT activity (Cao et al., 2007).

High temperature and water stress have been reported to decrease plant photosynthetic rate which is closely related to N metabolism (Jiang et al., 2021; Ru et al., 2022). The activities of NR and GS/GOGAT were reduced in the leaves of hemp seedlings under drought stress, however, the activity of glutamate dehydrogenase (GDH) was increased (Jiang et al., 2021). This is another pathway of N assimilation located in the plant cytosol and mitochondria (Kishorekumar et al., 2020) which is overexpressed under different stress conditions (Tercé-Laforgue et al., 2004). Similarly, heat stress significantly reduced the NR and GS activity of wheat plants and was more pronounced under the combined effect of heat and drought stress (Ru et al., 2022).

Soil contamination with heavy metals can influence plant metabolic processes positively or negatively depending on the contaminant and concentration in the soil (Mokhele et al., 2012). For instance, a low level of lanthanum promoted water movement and nutrient uptake as well as N assimilation in soybean seedlings (Cao et al., 2007), whereas cadmium reduced NO_3^- uptake (Gouia et al., 2000). A high aluminium

concentration in soil solution restricted NO_3^- uptake and inhibited NR activity in sunflower plants (Mokhele et al., 2012). Soil contamination with nickel decreased NR, NiR and Fd-GOGAT activity in wheat shoots while GS activity was not influenced (Gajewska and Skłodowska, 2009). Additionally, salinity stress causes several impairments in plants, including nutrient uptake with increased uptake of sodium (Na^+) over other nutrients, including P, N, calcium (Ca^{2+}), magnesium (Mg^{2+}), and potassium (K^+) (Shao et al., 2023). Reduction in GS activity has also been reported in wheat plants (Wang et al., 2007).

1.3 Mitigating nitrogen losses from fertilisers

1.3.1 Nitrogen losses in the environment

Excess application of N fertilisers in agriculture remains the main cause of increased reactive N (Nr) losses, such as N_2O and NH_3 emissions and N leaching, with detrimental impacts on the global N cycle (Albanito et al., 2017). It contributes to air and water pollution, climate change and stratospheric ozone depletion (Kanter et al., 2015). Pollution from excess N has exceeded the safe ‘planetary boundary’ threshold (Rockström et al., 2009).

1.3.1.1 Contamination to water bodies and soil

The unintended N losses from agricultural soils have resulted in loadings of 39-95 Tg N yr^{-1} and 40-66 Tg N yr^{-1} in fresh and coastal waters, respectively (Albanito et al., 2017). The water quality of several rivers in China has been negatively impacted by fertiliser use and industrial wastewater discharge (Chen et al., 2014). Similarly, most African coastal areas experience groundwater pollution from NO_3^- and other contaminants, principally due to the disposal of untreated industrial, domestic, and agricultural wastes and leached organic and inorganic fertilisers and pesticides (Elhaddad and Al-Zyoud, 2017).

Groundwater is commonly used as a potable water source. However, there is a raising concern regarding NO_3^- presence in groundwater globally (Abascal et al., 2022), making potable water supplies unsuitable for human and livestock consumption and threatening marine life (Khan et al., 2018). This is a critical issue as NO_3^- input to surface water can occur via groundwater discharge through baseflow into rivers and streams (Ju et al., 2006; Van Camp et al., 2021). Thus, preventing the deterioration of surface water quality also

requires preventing groundwater contamination (Khan et al., 2018). Groundwater quality monitoring in the highland agricultural watersheds in Ethiopia indicated that NO_3^- levels increased during the rainy season (Akale et al., 2018). This was linked to greater NO_3^- leaching of applied N fertilisers, with >1200 mm of rainfall occurring during the cropping season (Chand et al., 2011). In contrast, dry conditions are not favourable for the deep infiltration of nutrients below the plant root zone (Akale et al., 2018).

In soils, excessive application of N fertilisers can contribute to soil acidification (accumulation of H^+ ions from oxidation of NH_4^+) which can cause toxicity or a deficiency in the availability of some nutrients (Khan et al., 2018). Heavy metals (e.g., copper, lead, nickel, cadmium, copper, and zinc), for instance, become mobile at low pH, hence facilitating uptake by plants and entry into the food chain (Wei et al., 2020). Soil acidification from fertiliser use can also alter the soil microbial community which is pH dependent, and high acidity or too basic soil can be lethal to some microorganisms, such as symbiotic organisms (Zhang et al., 2015). As reported by Mondal et al. (2020) who observed a reduced rhizobium population in peanuts with a high N application rate.

1.3.1.2 Contamination to the air

Air pollution is the primary cause of respiratory diseases (WEF, 2018) and contributes to ozone depletion and global warming (Afolabi et al., 2019). The major air pollutants are carbon monoxide (CO), sulphur nitrate, particulate matter, and Nr gases (Afolabi et al., 2019). Globally, Nr emissions to the air from anthropogenic activities are dominated by agricultural activities, and the combustion of fossil fuels and biofuels (Galloway, 2003).

Reactive N, in the form of N_2O , contributes to climate change (Jia et al., 2019) and is the principal ozone-depleting gas (Galloway, 2003). Agriculture, forestry, and other land uses are the main anthropogenic sources of N_2O (Jia et al., 2019), with global emissions estimated at 5.3 Tg N_2O yr^{-1} of which agriculture accounts for 66% (industry, energy, and transport 15%, biomass burning 11% and other sources such as wastewater and aquaculture 3%; Davidson and Kanter, 2014). Thus, agriculture represents the primary source of anthropogenic N_2O emissions (Davidson and Kanter, 2014; Jia et al., 2019) deriving from the use of inorganic N fertilisers and manures (Jia et al., 2019), crop residues, exploitation of organic soils, and biological N fixation (Davidson and Kanter, 2014). A study conducted by Albanito et al. (2017) using datasets for the tropics and sub-

tropics revealed that Central and South America had the highest fertiliser-induced net emissions ($3 \pm 5.1 \text{ kg N ha}^{-1}$), followed by Australia > Asia > Africa. Compared to other regions, Africa's low contribution to global N_2O emission may be related to a lower rate of applied N fertilisers, with plants taking up more than was added and thus mining soil N and to the soil conditions (Hickman et al., 2014).

Among air pollutants, NH_3 is the most abundant alkaline gas in the atmosphere. It plays an important role in ecosystem N cycles, the neutralisation of acids in the air and the formation of fine particulate matter (Schiferl et al., 2014). The major sources of atmospheric NH_3 are agricultural activities, including animal husbandry, especially concentrated animal feeding operations, and N fertiliser use (Adon et al., 2016; Riddick et al., 2016). Ammonia emissions increase with increasing soil/source N content and pH, and with temperature (Riddick et al., 2016). Xu et al. (2019) suggested that temperature in relation to the timing of agricultural operations was the dominant factor influencing NH_3 emissions from agricultural systems. For instance, they found that in Asia, estimated NH_3 emissions were greatest during summer and intra-annual NH_3 emissions were highly correlated to dates and rates of N fertiliser application and cultivation periods (Xu et al., 2019). In contrast, spring accounted for about 60% of annual NH_3 emissions in North America and Europe due to high fertiliser use for corn and winter/spring wheat (Xu et al., 2019). In dry savannas, maximum N emissions are found at the beginning of the rainy season (both for reduced and oxidised compounds) due to the acceleration of microbial activity caused by increased soil moisture (Delon et al., 2012). Globally, the major crops associated with NH_3 emissions are rice, corn, and wheat, which receive >50% of the global synthetic N fertiliser; they accounted for about 65% of total NH_3 emissions during 1961-2010 (Xu et al., 2019). Pinder et al. (2004) indicated that the greatest uncertainty in NH_3 emissions is attributable to farming practices. Ammonia loss from applied N results in low fertiliser-N use efficiency, posing a substantial financial cost to farmers (Carswell et al., 2019; Pan et al., 2016).

Increasing food demand leads to intensified agricultural production, particularly in developing countries, with agricultural GHG and NH_3 emissions also expected to increase from this source (Albanito et al., 2017). Therefore, it is highly desirable to adopt

mitigation strategies that could effectively lower the contribution of agricultural sources pollution without compromising the growth and yield of crop plants (Khan et al., 2018).

1.3.2 Nitrogen use efficiency

1.3.2.1 Concept and common indices

Sustainable fertiliser use is critical for ensuring food security and for reducing the impacts of fertiliser use on the environment (Chianu et al., 2012) and society. Increasing the proportion of N fertiliser transferred into crop and forage yields has been instrumental in improving fertiliser NUE and crop productivity while reducing unintended environmental pollution (Ladha et al., 2020; Zhang et al., 2015). In general, 50-70% of N fertilisers applied to soil is lost into the environment (Lawrencia et al., 2021; Tyagi et al., 2022; Wu, 2011) although, in Sub-Saharan Africa (SSA), NUE often exceeds 100%, indicating that where fertilizer use is limited soils are mined of nutrients (Edmonds et al., 2009).

Nitrogen use efficiency is globally accounted as the ratio of N output in desired product (e.g., harvestable crop components, livestock products) to N inputs (from all or specified sources e.g. including N₂ fixation, fertiliser, manure, deposition, etc.; Ladha et al., 2020; Zhang et al., 2020). Several agronomic indices with different measurement methods have been used to determine fertiliser NUE (Dobermann, 2007; Ladha et al., 2020). The most common indices (Table 2) used in agronomic research for capturing best management practices include apparent crop recovery efficiency (RE_N), physiological efficiency (PE_N), internal utilisation efficiency (IE_N), agronomic use efficiency (AE_N), and partial factor productivity (PFP; Dobermann, 2007).

Table 1-2: Common N use efficiency indices

(Summarised from Dobermann, 2007)

Indices	Formula	Interpretation
RE _N	$= \frac{N_F - N_C}{N_R}$	RE _N is the kg augmentation in N uptake per kg N applied. It can also be expressed in percentage. RE _N is influenced by the relationship between plant N requirement and N available from fertiliser, but also by fertiliser management 4R's (i.e., right source, right rate, right time, and right place) and variety, crop management, environmental conditions.

PE_N	$= \frac{W_F - W_C}{N_F - N_C}$	<p>PE_N refers to kg yield gain per kg raise in N uptake from applied fertiliser.</p> <p>It reflects the plant potential to produce yield from the N taken up, therefore relate to factors impacting N uptake and assimilation such as genotype, soil and environmental condition and crop and fertiliser management.</p>
AE_N	$= \frac{W_F - W_C}{N_R}$ <p>or</p> $= RE_N \times PE_N$	<p>AE_N is considered as a kg yield gain per kg N applied.</p> <p>AE_N is influenced by crop, water and fertiliser management practices and other factors affecting RE_N and PE_N.</p>
IE_N	$= \frac{W_F}{N_F}$	<p>IE_N refers to the kg yield per kg N uptake.</p> <p>IE_N shows plant ability to convert into economic yield the N taken up from all available N sources (fertiliser, soil residual N, etc.).</p> <p>IE_N varies depending on genotype difference, soil and environmental condition, crop, water and fertiliser management practices.</p>
PFP	$= \frac{W_F}{N_R}$ <p>or</p> $= \frac{W_C}{N_R} + AE_N$	<p>PFP refers to kg reaped product per kg N fertiliser applied.</p> <p>PFP is more useful to farmers as it informs of the efficient use of soil residual N and fertiliser N applied. It also relates to plant AE_N.</p>

In the formula column, N_F and N_C are the total N uptake of whole plant at maturity from fertilised plots and from plots that received zero N fertiliser (kg ha^{-1}), respectively, and N_R is the rate of N applied (kg N ha^{-1}). W_F and W_C are the harvested plant weight from N fertilised plots and from plots that received zero N fertiliser (kg ha^{-1}), respectively.

Other indices capture more variations within and between agroecosystems such as tracing NUE using stable N isotopes, ecology and system based NUE (Congreves et al., 2021). The nutrient budgeting approach, also called soil nutrient balance (Zhang et al., 2020), is also useful for assessing nutrient use efficiency (Chianu et al., 2012; Zhang et al., 2020), as well as potential environmental pollution related to the application of nutrients (Alley and Vanlauwe, 2009). It can also provide guidance to farmers in developing nutrient management interventions for individual crops and fields (Chianu et al., 2012). Using nutrient budgets is a promising progression towards more balanced systems. The nutrient budgets highlight that when inputs are in excess of crop nutrient

removal, there is high probability for environmental contaminations from the excess nutrients, especially N via volatilisation or leaching (Chianu et al., 2012).

1.3.2.2 Nitrogen use efficiency targets

In countries where intensive food production is a reality, the efficiency of applying nutrients has been considered a good agro-environmental indicator (Brentrup and Pallière, 2010). Chang et al. (2021) reported that enhancing NUE is the most promising N mitigation approach. Although, enhanced NUE doesn't always translate to reduction in N losses, it is a key step towards Nr mitigation while sustaining agricultural productivity (Norton et al., 2015). A desirable outcome of enhancing NUE for farmers and the environment would be an increase in crop yields and a reduction in N mining from low N input, and N surplus from high N input (Norton et al., 2015). Experiences have revealed that maintaining crop N recovery efficiency slightly below or up to 70% secures efficient crop productivity and attenuates potential environmental pollution and land degradation (Sutton et al., 2013). Zhang et al. (2015) suggested a global target for NUE of 70% by 2050, although targets might vary depending on cropping systems and national policies in place.

However, with climate change scenarios of extreme climate events, including warmer and dryer weather conditions with frequent drought and heavy rainfall (IPCC, 2023), it becomes crucial to implement adaptation strategies to reduce agroecosystem vulnerability. Extreme weather and climate events have already exposed many vulnerable communities to food and water precarity (IPCC, 2023). Severe impacts have been recorded in several regions (IPCC, 2023) and a gradual and substantial increase in recurrence of water scarcity (drought) with warming is expected in the Mediterranean region, most of Africa, West and Southern Asia, Central America, and Oceania (Naumann et al. 2018). In Africa, for instance, increasing temperatures and heat waves, and changes in precipitation patterns has greatly impacted vulnerable communities, especially food producers dependent on rainfed agriculture and pastoralists (IPCC, 2023). Thus, improving fertiliser NUE in agriculture requires controlling factors that impact the soil N availability, uptake and efficient use in economic yields by plants. This involves promoting the adoption of appropriate technologies and management practices at the farm

scale and improving plant breeding for adapted crop varieties (Ferguson, 2015; Newell Price et al., 2011; Zhang et al., 2015).

1.3.3 Management-based solutions

1.3.3.1 Soil and fertiliser management

Several strategies have been introduced to mitigate water pollution, GHG and NH_3 volatilisation from agricultural land, and improve soil health and quality (Newell Price et al., 2011). Regarding fertiliser NUE improvement in cropping systems, the common practices include, but are not limited to, management factors such as deep placement (e.g., drilling or injection, incorporation, fertigation) of the N fertiliser into the soil which reduces the proportion of NH_3 loss compared to surface broadcasting; essentially because the N fertiliser is less affected by environmental conditions (Dari et al., 2019; Shafreen et al., 2021). The “four rights” (4Rs) strategy, consisting of applying the right source of N fertiliser, at the right rate, at the right time and in the right place (Snyder et al., 2014) is essential for improving fertiliser NUE (Singh et al., 2021). Further strategies that could improve NUE and soil health include foliar applications of N and adopting resource conservation practices such as conservation agriculture (e.g., zero tillage, permanent beds, and mulching; Burgess et al., 2002; Tyagi et al., 2022). Several authors have reported the profitability of conservation agriculture in rice-wheat systems in terms of yield, production cost, efficient use of resources and environmental quality (Bhatt et al., 2021; Jat et al., 2019). Additionally, integrated nutrient management, consisting of the combined use of inorganic fertilisers and manure, or other organic nutrient sources (e.g., compost, green manure, digestates) to improve soil fertility health and productivity (Garai et al., 2021). Integration of organic sources provides both macro- and micro-nutrients to plants and improves the soil physical, chemical, and biological properties (Garai et al., 2021). The adoption of precision agriculture technologies to reduce labour requirement, optimise the management of fertilisers and irrigation and increase profitability (Bhatt et al., 2021; Singh et al., 2020; Tyagi et al., 2022) is an effective way to enable site-specific management (Singh et al., 2021). Furthermore, N management challenges in organic farming systems can also be tackled using the same practices and technologies (Dobermann, 2007; Newell Price et al., 2011).

1.3.3.2 Crop and water management

Improving fertiliser NUE requires optimising crop uptake and internal plant N utilisation (Dobermann, 2007). One of the promising modern advances is the genetic improvement of crops for better adaptation to environmental stresses (e.g., heat stress, water scarcity, water-logging, salt stress, ion toxicity and other abiotic stresses like wind) and greater utilisation efficiency of farm inputs (Sabagh et al., 2021). Adopting short-duration paddy rice cultivars has been shown to be a good option in conservation agriculture, as they have low fertiliser and irrigation requirements (Bhatt et al., 2021; Tahat et al., 2020). Continuous cropping of the same crop can cause specific nutrient depletion leading to yield reduction (Bhatt et al., 2021). Therefore, crop diversification through crop rotations, intercropping, managing crop density and sowing dates are potential options for increasing NUE (Tyagi et al., 2022). Plant inoculation with arbuscular mycorrhizal fungi, cyanobacteria, and beneficial nematodes are potential practices to optimise plant nutrient and water use efficiency and improve tolerance to environmental stresses (Tahat et al., 2020).

Water-saving technologies to maximise the efficiency of water use can also increase NUE (Drechsel et al., 2015). Limited or excess water supply reduces NUE and induces N losses through volatilisation, leaching (Drechsel et al., 2015) and denitrification. Practices include deficit irrigation, low-pressure irrigation, subsurface drip irrigation, drip irrigation under plastic covers, furrow irrigation, rainfall harvesting and in-situ water conservation techniques (Drechsel et al., 2015). Deficit irrigation is adopted in west Asia, north Africa, and northern China (Drechsel et al., 2015; Oweis and Hachum, 2009). Soil matric potential-based irrigation strategies, measuring soil water availability to plants, are among the appropriate techniques for adaptation to climate change (Bhatt, 2020; Bhatt et al., 2021; Singh et al., 2014).

1.3.4 Fertiliser-based solutions

Optimising fertiliser NUE to augment food production and significantly reduce gas and leaching losses is a priority in low input and intensified crop production areas (Hawkesford et al., 2014). Enhanced efficiency fertilisers (EEF) are among the promising mitigation technologies to reduce excess N and the concurrent negative effects on the environment (Suman et al., 2023). These novel fertilisers mainly include slow or

controlled-release fertilisers, and stabilised N (which contain N cycle inhibitors) fertilisers (Halvorson et al., 2014; Singh et al., 2021). The principal advantages of using EEF include extended availability of nutrients, reduced N application frequency, economic gain and reduction in N losses (Behin and Sadeghi, 2016; Dimkpa et al., 2020). The disadvantages of EEF relate to their elevated cost, limited availability and application complexity (Fu et al., 2018; Suman et al., 2023).

1.3.4.1 Slow and controlled-release fertilisers

Compared to conventional fertilisers, slow and controlled-release fertilisers gradually release macro- and micro-nutrients for plant use (Singh et al., 2021; Suman et al., 2023). Slow-release fertilisers are conventional soluble fertilisers coated with biodegradable low-solubility polymers (organic and inorganic) that discharge nutrients through microbial or chemical disintegration (Dimkpa et al., 2020; Lawrencia et al., 2021). Controlled-release fertilisers are conventional soluble fertilisers coated or encapsulated with inorganic or organic constituents (Dimkpa et al., 2020; Suman et al., 2023), which provide a physical barrier that regulates water penetration and the rate of discharge of loaded nutrients (Lawrencia et al., 2021; Trenkel, 2010). The common inorganic constituents include phosphogypsum, bentodine, sulphur and the organic polymers can be synthetic (polyethene, polyurethane, alkyd resin, etc.) or natural (chitosan, starch, cellulose, etc.; Haydar et al., 2024; Lawrencia et al., 2021; Trenkel, 2010). Slow and controlled-release fertilisers should meet three conditions predefined as follows: release of $\leq 15\%$ of the N in 24 h; release of $\leq 75\%$ in 28 days; and release of $\geq 75\%$ at the desired time (Suman et al., 2023; Trenkel, 2010). The factors driving the rate, pattern and duration of nutrient release can be fully controlled during preparation, particularly for controlled-release fertilisers (Haydar et al., 2024; Liu et al., 2023). Recommended fertiliser application rates can be reduced by over 20% using slow and controlled-release fertilisers (Trenkel, 2010). Furthermore, slow and controlled-release nano-fertilisers have recently emerged to improve NUE and yield, reduce N losses and resolve other soil nutrient deficiencies (Haydar et al., 2024). They are also made from complex polymer materials and coatings or encapsulated, but on the nanoscale (Haydar et al., 2024). Nanoparticles of different compositions (NPK, $\underline{\text{C}}$, Mg, Fe, Si, ZnO) have been reported to enhance plant growth, yield, N uptake and NUE in wheat, rice, maize, peas and vegetables (Bala et al., 2019; Haydar et al., 2024; Zhao et al., 2021). Slow and controlled-release fertilisers

increased rice N uptake and yield by 11% and 6%, respectively, compared to conventional fertilisers (Liu et al., 2023). A meta-analysis by Zhang et al. (2019) reported reductions in NH_3 volatilisation, N_2O emission and leached N losses by 39%, 24% and 27%, respectively, using a controlled-release urea.

However, there are concerns regarding the phytotoxicity of nanoparticles, as plant species respond differently to applied doses and types of nanomaterials (Liu et al., 2022). Additionally, some studies have reported no beneficial effect of nano-fertilisers on plant development (Kumar et al., 2019). Furthermore, slow-release fertilisers may cause nutrient deficiencies when nutrients are not released to suit plant-specific growth nutrient requirements (Liu et al., 2023; Trenkel, 2010). The use of sulphur-coated urea has been reported to decrease soil pH, with potential impacts on other nutrient availability such as Ca and Mg (Lawrencia et al., 2021; Liu et al., 2023). Low-degradable synthetic polymer coatings may introduce other forms of pollution (Lawrencia et al., 2021) such as microplastics, which are a major soil contaminant (Uwamungu et al., 2022). Large-scale field studies on nano-fertiliser use in agriculture are required to get more insight into their potential benefits (Tyagi et al., 2022) and long-term effects on the soil-plant-water-atmosphere and global food system under different management and environmental conditions (Kumar et al., 2019).

1.3.4.2 Influencing factors of slow and controlled-release fertilisers

Temperature and Moisture

Nutrient release from slow-release fertilisers is not well controlled as it depends on microbial activity, which is highly influenced by soil temperature and moisture conditions (Liu et al., 2023). Al-Samarrai and Hamzah (2021) reported an increase in nutrient release from slow-release fertilisers with increasing temperature, with release times of 40, 34 and 17 days at 35, 45 and 55 °C, relative to 47 days at room temperature. Fan and Li (2010) also found a faster release of N with increasing soil temperature (20, 25 and 30°C) and greater NH_3 volatilisation from slow-release fertilisers (Fan et al., 2011). Nutrient release from controlled-released fertilisers has three phases: lag period, constant release and decay period (Trenkel, 2010). The lag period corresponds to water infiltration through the coating or capsule, which raises the internal pressure to saturation, resulting in a constant release of nutrients. The decay stage releases most coated or encapsulated nutrients

(Lawrencia et al., 2021). The increase in temperature shortens the duration of the lag period, pore size and swelling which increases the rate of nutrient release (Du et al., 2006; Lawrencia et al., 2021) because of the increase in nutrient diffusion, which is reportedly rapid at temperatures $>25^{\circ}\text{C}$ (Bi et al., 2020). Too much water pressure could cause rupturing of the coating, leading to an instant release of the nutrients within (Azeem et al., 2014; Haydar et al., 2024). Additionally, nutrient release may be prolonged after the crop cycle due to the tailing effect that occurs when 80-85% of the nutrients have been released (Lawrencia et al., 2021; Shaviv, 2001).

Soil factors

Differences in soil physicochemical characteristics influence the efficiency of controlled-release fertilisers. The higher soil pH and organic C content of loamy soils favour microbial N mineralisation (Gioacchini et al., 2006). Fan and Li (2010) found significantly greater N-release rates from slow-release fertilisers in a loamy soil compared to a sandy soil. However, the cumulative NH_3 emissions from the slow-release fertiliser was lower from the loamy soil relative to the sandy soil, due to rapid nitrification of the released NH_4^+ in the loamy soil, reducing the NH_4^+ retention time in soil, which was longer in the sandy soil (Fan et al., 2011). The meta-analysis of Zhang et al. (2019) revealed that the efficacy of controlled-release fertilisers in mitigating NH_3 and N_2O emissions and N leaching was more pronounced in light-textured or low organic matter content soils. Slow and controlled-release fertiliser coating thickness, uniformity, particle size and material composition can influence nutrient release (Firmanda et al., 2023; Haydar et al., 2024; Lawrencia et al., 2021). Generally, conditions favouring increased soil microbial activity (as described above) can cause excess nutrient release with potential leaching losses, particularly after the cropping cycle (Trenkel, 2010; Zhang et al., 2019).

1.3.4.3 Nitrogen cycle inhibitors

Nitrogen cycle inhibitors, also called N fertiliser stabilisers, operate by inhibiting the biological activity of specific soil microflora or biochemical catalysts (Suman et al., 2023; Trenkel, 2010). Commonly used N cycle inhibitors are urease inhibitors (UI), which temporarily block the active site of the urease enzyme, delaying urea hydrolysis in soil and decreasing emissions of NH_3 (Suman et al., 2023). Nitrification inhibitors (NI)

temporally inhibit the enzyme ammonia monooxygenase, which is present in nitrifying bacteria and catalyses NH_4^+ oxidation into NO_2^- (Suman et al., 2023), thereby decreasing N_2O emission and leaching of NO_3^- (Byrne et al., 2020; Freeman et al., 2020). There are a variety of compounds used as UI and NI, however, commercially available UI include N-(n-butyl) thiophosphoric triamide (NBPT), N-propyl- thiosphosphoric- triamide (NPPT) and N-(2-nitrophenyl) phosphoric triamide (2-NPT). Commercially available NI include dicyandiamide (DCD), 3,4-dimethyl-1H-pyrazole phosphate (DMPP), 4,5-dimethylpyrazole-1-yl-succinic acid (DMPSA) and nitrapyrin (Byrne et al., 2020). These UI and NI are classified as organic chemicals (Byrne et al., 2020). The UI and NI can be incorporated with N fertilisers separately and combined double inhibitor products to target NH_3 and N_2O emissions and NO_3^- leaching simultaneously (Duff et al., 2022; Hofman and Cleemput, 2004). Urease inhibitors have been reported to reduce the emission of NH_3 by up to 84% (Byrne et al., 2020) and where UI influence on yield was observed, the yield increase varied from 0.8 to 10.2% depending on the crop species (Cantarella et al., 2018). Nitrification inhibitors have been reported to have lower yield increase potential compared to UI (Abalos et al., 2014); however, for NUE, an increase of up to 12.9% on average was reported for UI and NI or their combination (Abalos et al., 2014). Several studies found no significant effect of NI on yield, N uptake or leaching losses (Misselbrook et al., 2014). Nevertheless, on average, NI can reduce N_2O emission from applied fertilisers by over 65% (Misselbrook et al., 2014; Ruser and Schulz, 2015), with reduction in soil NO_3^- concentration and potential leaching losses (Guardia et al., 2018). Both UI and NI can reduce NO_3^- concentration in soil, lowering the substrate needed for denitrification processes (Abalos et al., 2014). However, the application of NI has often been found to induce an increase in NH_3 emissions due to the prolonged availability of NH_4^+ in soil solution (Abalos et al., 2014).

Plant uptake UI, particularly NBPT, with subsequent inhibition effects on plant urease activity and some studies reporting disruption of N metabolism (Ariz et al., 2012; Cruchaga et al., 2011). Byrne et al. (2020) reported that phenyl phosphorodiamidates inhibitor compounds have a stronger inhibitory effect on plant urease than microbial urease. Similarly, plant uptake of NI has been reported, particularly for DCD, DMPP and DMPSA, with DCD and DMPP causing necrosis on the leaf margins under high concentration (Byrne et al., 2020; Cameron et al., 2013). The presence of DCD in milk

powder has raised food safety concerns regarding its use in dairy pastures (Rodrigues et al., 2018). No impacts of UI or NI have been found on the function and abundance of non-targeted microbes (Duff et al., 2022), and there is little evidence of long-term effects on the N cycling microbial community (Ruser and Schulz, 2015). Nevertheless, some recent studies have found that the influence of NBPT on plant N metabolism activates compensation mechanisms, which may enable certain plants to enhance their tolerance to some abiotic stresses (Gou et al., 2017; Haque et al., 2022). More studies are needed to provide insight into the possible contribution of these inhibitors to plant stress tolerance.

Other UI include metal complex inhibitors, with different degrees of urease inhibition (i.e., $\text{Ag}^+ \sim \text{Hg}_2^+ > \text{Cu}_2^+ > \text{Cd}_2^+ > \text{Co}_2^+ > \text{Ni}_2^+ > \text{Zn}_2^+ = \text{Sn}_2^+ = \text{Mn}_2^+ = \text{Pb}_2^+$) due to their reactions with sulfhydryl groups of ureases forming insoluble sulfide metals, which can deactivate urease at sufficient concentrations (Byrne et al., 2020; Cantarella et al., 2018). Different combinations of these metals used to supply plants with micronutrients have been reported to lower NH_3 losses from urea applications (Dimkpa et al., 2020) as they can have an inhibitory effect on certain soil enzymes (Liu et al., 2022; Wang et al., 2018). Utilising Ca and Mg-rich minerals (e.g., limestone, gypsum, dolomites) with urea has been reported to reduce N losses (Suman et al., 2023). Biological compounds with NI properties have been isolated from various plants (e.g., pasture grasses and field crops) with promising potential (Norton and Ouyang, 2019; Wang et al., 2021). Organic products such as neem (oil, seed cake) have been reported to have NI properties and are widely used in India (Suman et al., 2023), but may be associated with increased urease enzyme activity (Kizilkaya et al., 2015) and greater NH_3 volatilisation.

1.3.4.4 Influencing factors of nitrogen cycle inhibitors

The efficacy of fertilisers incorporating N cycle inhibitors in improving NUE, yield and mitigating N losses is influenced by other factors including temperature, pH, moisture, ionic interaction of soil and the microbial activity of soil (Haydar et al., 2024; Suman et al., 2023).

Temperature

The persistence of N cycle inhibitors in soil depends on their biodegradation rate, which drives their efficacy in N-loss mitigation (Byrne et al., 2020). The half-life of the UI

NBPT under storage has been found to decrease with increasing storage temperature (4, 25 and 34°C; Watson et al., 2008). The rate of UI degradation in storage may be related to the method of UI application, being faster when the UI is coated on urea granules than when mixed with the urea melt before granulation (Soares, 2011). However, despite the half-life reduction of UI stored for a long period at high temperatures (>20°C), Cantarella et al. (2016) still observed a reduction in NH₃ volatilisation when it was applied to soil, although less of a reduction than with UI stored at lower temperatures. Similarly, increasing soil temperature augments the degradation rate of UI and NI in soil (Chen et al., 2008), with increased microbial mineralisation of UI and NI (Guardia et al., 2018) strongly influencing the inhibition effect (Menéndez et al., 2012, McGeough et al., 2016). For instance, nitrapyrin has been reported to degrade within 30 days in warm soil, whereas it is stable for much longer in the winter and autumn when soil temperatures are reduced (Byrne et al., 2020). Soil temperatures above 16°C reduced the efficacy of both DCD and DMPP (Chen et al., 2008). The mineralisation of DCD and DMPP in soil was greater at 30°C relative to 10 and 20°C, decreasing the NI half-life as temperature increased (Guardia et al., 2018). A soil incubation study observed a reduction of DCD half-life by up to 79% between 5 and 25°C (McGeough et al., 2016).

Moisture

Irrigation or rainfall events occurring shortly after urea application have increased UI efficacy in reducing NH₃ volatilisation from urea (by up to 78%), by facilitating urea and UI displacement into the soil (Cantarella et al., 2008). Leaching of DCD after rain events has been reported by Cahalan et al. (2015) from grassland soils, which potentially affects the duration of the inhibition effect (Guardia et al., 2018). Menéndez et al. (2012) reported that soil moisture influenced DMPP molecule residence time in soil, with a higher residence time at 80% water-filled pore space (WFPS) compared to 40% WFPS, which may be related to O₂ availability enabling DMPP degradation. However, the efficiency of DMPP was greater at 40% WFPS as nitrification would be the main N₂O production pathway. Furthermore, the efficiency of DMPP increased with increasing temperature 10, 15 and 20°C (from 17% up to 42%) at 40% WFPS, whereas at 80% WFPS, the opposite was true (from 45% to 23%). This would be caused by the effect of high temperature on

the N₂:N₂O emission ratio from denitrification, shifting toward the emission of N₂ rather than N₂O as the end product (Maag and Vinther, 1996; Menéndez et al., 2012).

Soil factors

High and low pH accelerates UI degradation due to chemical hydrolysis and microbial degradation (Byrne et al., 2020; Engel et al., 2015; Li et al., 2006), with one study reporting a reduction in the half-life of NBPT from 92 days at pH 7.0 to 16 days at pH 11.0 and to 58 min at pH 3.0 (all at 25°C; NICNAS, 2011). Silva et al. (2017) reported lower UI efficiency in reducing N losses under low soil pH than in neutral and alkaline soil. Linquist et al. (2013) reported no benefit of UI and NI in rice systems on yield and N uptake under a soil pH of 6.0. In their meta-analyses, Abalos et al. (2014) demonstrated that use of UI and NI enhanced crop productivity and NUE significantly more at soil pH ≤ 6 than in neutral to alkaline soil. Guardia et al. (2018) found lower efficacy of DCD and DMPP in alkaline soil (pH 7.6) and lower CEC, clay and organic matter content than acidic soil (pH 6.0). In contrast, Lei et al. (2022) found higher efficacy of DMPP in alkaline soil (pH ≥ 8), compared to acidic soil.

Furthermore, inhibitor efficiency can be influenced by absorption into soil colloids (e.g., clays and organic matter) and immobilisation by non-targeted microorganisms (Guardia et al., 2018; Hoef, 1984). For instance, higher recovery of DCD in soil relative to DMPP was reported by Guardia et al. (2018) and was related to the greater sorption of DCD to the soil matrix. Crop residue or organic matter incorporation in the soil can reduce UI and NI efficacy as more plant biomass added to the system will increase microbial activity (Cantarella et al., 2018; Silva et al., 2017). Soil physicochemical properties also affect NI and UI inhibition effects (Abalos et al., 2014; Guardia et al., 2018; McGeough et al., 2016). For instance, Guardia et al. (2018) found greater mineralisation of DCD in an acidic UK soil than in an alkaline Spanish soil. In contrast, DMPP mineralisation was not influenced by soil type. Moreover, regarding temperature and soil type interaction, NI efficacy was lower at 20°C in the acid soil and lower at 30°C in the alkaline soil (Guardia et al., 2018).

Additionally, the type and rate of fertilisers and inhibitors can determine the efficiency in different environments and soil conditions (Bell et al., 2015; Cantarella et al., 2018; Guardia et al., 2018; Misselbrook et al., 2014). For instance, DMPP has a lower degradability than DCD (Chaves et al., 2006) which will influence their efficacy under

similar conditions. In their study using permanent grassland and permanent arable soils, Guardia et al. (2018) reported a greater half-life of 635 days for DCD compared to 273 days for DMPP across both soils. The effect of UI and NI in mitigating N losses is reduced under high N supply (Bell et al., 2015; Silva et al., 2017). Additionally, the application of high concentrations of UI (>1000 mg NBPT kg^{-1} urea) has been reported to have a greater effect on reducing NH_3 losses but with no influence on crop yield (Cantarella et al., 2018; Silva et al., 2017). Soil texture influenced the effect of UI and NI on crop yield, with increased yield under fine-textured soils relative to medium or coarse-textured soils (Abalos et al., 2014). However, the meta-analysis of Silva et al. (2017) did not find an effect of soil texture on UI efficacy in reducing N volatilisation losses. Lei et al. (2022) reported from their meta-analysis that NI gave a potential reduction in N_2O emissions of 75% in medium soils (with lower soil organic matter) compared to 47% on average in coarse and fine-textured soils.

1.4 Summary

The growing world population continues to exert pressure on limited natural resources to produce food. The development of different production systems (cereal, vegetables, pastures, forestry, etc.) rely on fertiliser inputs to increase productivity from limited cultivatable land (Bumb, 1995). Excessive use of chemical fertilisers, principally, N fertilisers has contributed to unprecedented soil, water, and environmental degradation and climate change. The challenge of producing enough food without causing adverse impacts on the environment, requires careful management of N fertiliser inputs. Several agricultural practices (e.g., ferti-irrigation, crop rotations) and technologies (e.g., precision agriculture, slow and controlled-release fertilisers) have emerged to help meet the target of improving fertiliser NUE. Genetic improvement of crop varieties to increase yield potential and adaptation to biotic and abiotic stress has enhanced fertiliser NUE. Single or combined adoption of practices or technologies have proven to be efficient strategies in mitigating N losses through gaseous (e.g., NH_3 , N_2O), and hydrological (e.g., NO_3^- , NH_4^+) losses. Development of EEF namely slow and controlled-release fertilisers and N cycle inhibitors (UI and NI) is widely accepted as an effective mitigation strategy for N losses from soil microbial processes involved in the soil N cycle. Although, they could cause some impairments in the plant N cycle under certain conditions.

The efficacy of EEF is affected by multiple soil, climatic and management factors, and their interactions. Therefore, identifying the factors controlling the efficacy of EEF in N loss mitigation, crop production and NUE is crucial to promoting the development and utilisation of EEF. Faced with the projected climate change scenarios, temperature and soil water content may be important limiting factors on the effectiveness of N cycle inhibitors. However, authors like Menéndez et al. (2012) and Fan et al. (2022) reported that N cycle inhibitors would perform better in reducing N losses under conditions favourable for N loss (e.g., cold and wet and hot and dry conditions). Nevertheless, temperature and soil moisture for instance both have an impact on the lifespan of UI. The UI lifespan decreases with increasing temperature and low soil moisture can restrict UI solubility and the amount of inhibited urease enzyme. Although studies have observed an efficacy of UI to mitigate N losses under various climatic and environmental conditions, very few have investigated UI efficacy under combined effect of extreme high temperatures and extreme low soil moistures. Thus, there is a need to investigate the efficacy of N cycle inhibitors in reducing N losses and increasing NUE at temperatures above 25°C, greater than those often reported in the literature, and in a variety of soil and environmental conditions such as drought stress to assess their effectiveness under future extreme climate events. Drought events are expected to intensify with direct impacts on crops and agricultural sustainability (Furtak and Wolińska, 2023). Fertilisation with EEF, particularly urea treated with UI, showed potential to enhance crop tolerance to drought and increase its nutritional quality (Haque et al., 2022). Nevertheless, the limited literature on UI use under environmental stresses and the subsequent effect on crop performance does not cover all staple crops. Accordingly, the combined use of improved crop varieties with EEF to improve productivity and enhance crop adaptation to extreme events while reducing N losses from soil need to receive more attention. Additionally, the contribution to N losses in cropping systems sourced directly from plants needs better understanding, especially in systems receiving UI given the resulting impediments in plant N metabolism reported by several authors (Cruchaga et al., 2011; Artola et al. 2011). The identified knowledge gaps around the efficacy of UI under weather extremes and the influence of UI on crop tolerance to drought stress and on plant N assimilation and losses led to the elaboration of the research hypotheses stated below.

The hypothesis derived from this literature review are as follows:

Hypothesis 1

High temperature and low soil moisture exacerbate ammonia emissions from urea fertiliser, and this can be offset with the use of UI. However, these conditions will also reduce the duration over which the inhibitor is effective.

Hypothesis 2

Nitrous oxide is emitted via plant stomata during the assimilation of internal NO_3^- .

Hypothesis 3

The rate of N_2O emission from plants depends on the wheat genotype and the type and amount of N applied.

Hypothesis 4

Inclusion of UI with urea fertiliser affects N assimilation in wheat with potential impact on N_2O emission.

Hypothesis 5

Urea treated with UI improves fertiliser NUE in wheat under well-watered and drought stress conditions, despite N assimilation impairments induced by UI in leaves.

Hypothesis 6

Inclusion of UI with urea fertiliser enhances plant resilience to drought stress.

1.5 Aims and objectives

The overall aims of this thesis are to explore how the use of UI can mitigate N losses from urea fertiliser under warm and dry climatic conditions and enhance fertiliser NUE under abiotic stress and to determine plant contribution to N_2O emissions in the environment.

The following objectives were set to deliver against the overall aims:

Objective 1

Explore the efficacy of two UI in a range of temperatures and soil moistures in terms of reducing NH_3 emissions and whether this has an impact on N_2O emissions (relates to hypothesis 1).

Objective 2

Understand plant contribution to N_2O emission and the influence of UI and fertiliser types on plant leaf-N storage compounds and N_2O emission (relates to hypothesis 2, 3 and 4).

Objective 3

Determine the effect of UI on fertiliser NUE, under conditions of optimum soil moisture or drought, and on leaf-N storage compounds using wheat growth experiments (relates to hypothesis 5 and 6).

1.6 Structure of the thesis

This thesis is divided into 5 chapters including this introductory chapter (**Chapter 1**) which gives a general overview of the soil and plant N cycle and influencing factors. In addition, a review of the use of synthetic N fertiliser use in agriculture and their contribution to environmental pollution and GHG emissions is presented alongside a summary of mitigation strategies to improve NUE.

Chapter 2 addresses Objective 1 and presents a soil incubation study performed to determine the influence of high temperature and low soil moisture on the efficacy of two UI (NBPT, NPPT) to reduce NH_3 emissions and subsequent influence on N_2O emissions. This chapter has been published in the Journal of Environmental Management.

Chapter 3 relates to Objective 2 and investigates the possible contribution of plants to N_2O emissions in the environment and its formation pathway in the wheat leaves, as well as the influence of N fertiliser type (including urea with NBPT) and rate on leaf N assimilation and emission of N_2O using both soil and hydroponic experiments.

Chapter 4 addresses Objective 3 and determines the effect of NBPT on leaf N assimilation and the potential to enhance wheat tolerance to drought stress and improve fertiliser-NUE indices under drought stressed condition for three Spring wheat varieties in a pot experiment.

Chapter 5 synthesises and discusses the main findings from the objectives, highlights the thesis contribution to knowledge and proposes areas for future research.

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2 Effects of Hotter, Drier Conditions on Gaseous Losses from Nitrogen Fertilisers

Marieme Drame^{1,2,5}, Alison Carswell^{1*}, William Roberts¹, Jess Hood⁶, Martin Jemo³, Sigrid Heuer⁴, Guy Kirk², Mark Pawlett², Tom Misselbrook¹

¹Net-zero & Resilient Farming, Rothamsted Research, North Wyke, UK.

²Environment and Agrifood, Cranfield University, Cranfield, UK.

³Mohammed VI Polytechnic University Lot 660, Hay Moulay Rachid Ben Guerir, Morocco.

⁴National Institute of Agricultural Botany, 93 Lawrence Weaver Road, Cambridge, UK.

⁵Sustainable Soils and Crops, Rothamsted Research, Harpenden, UK.

⁶Intelligent Data Ecosystems, Rothamsted Research, Harpenden, UK

*Correspondence: alison.carswell@rothamsted.ac.uk

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2.1 Abstract

Global warming is expected to cause hotter, drier summers and more extreme weather events including heat waves and droughts. A little understood aspect of this is its effects on the efficacy of fertilisers and related nutrient losses into the environment. We explored the effects of high soil temperature ($> 25^{\circ}\text{C}$) and low soil moisture ($< 40\%$ water filled pore space; WFPS) on emissions of ammonia (NH_3) and nitrous oxide (N_2O) following application of urea to soil and the efficacy of urease inhibitors (UI) in slowing N losses. We incubated soil columns at three temperatures ($15, 25, 35^{\circ}\text{C}$) and three soil moisture contents ($20, 40, 60\%$ WFPS) with urea applied on the soil surface with and without UIs, and measured NH_3 and N_2O emissions using chambers placed over the columns. Four fertiliser treatments were applied in triplicate in a randomised complete block design: (1) urea; (2) urea with a single UI (N-(n-butyl) thiophosphoric triamide (NBPT)); (3) urea with two UI (NBPT and N-(n-propyl) thiophosphoric triamide; NPPT); and (4) a zero N control. Inclusion of UI with urea, relative to urea alone, delayed and reduced peak NH_3 emissions. However, the efficacy of UI was reduced with increasing temperature and decreasing soil moisture. Cumulative NH_3 emission did not differ between the two UI

treatments for a given set of conditions and was reduced by 22–87% compared with urea alone. Maximum cumulative NH_3 emission occurred at 35°C and 20% WFPS, accounting for 31% of the applied N for the urea treatment and 25%, on average for the UI treatments. Urease inhibitors did not influence N_2O emissions; however, there were interactive impacts of temperature and moisture, with higher cumulative emissions at 40% WFPS and 15 and 25°C accounting for 1.85–2.62% of the applied N, whereas at 35°C there was greater N_2O emission at 60% WFPS. Our results suggest that inclusion of UI with urea effectively reduces NH_3 losses at temperatures reaching 35°C, although overall effectiveness decreases with increasing temperature, particularly under low soil moisture conditions.

Keywords: Nitrogen fertiliser; urease; greenhouse gas; ammonia mitigation

2.2 Highlights

- Urease inhibitors (UI) efficacy was influenced by temperature and soil moisture.
- Ammonia losses were reduced by UI under warm and dry conditions.
- Temperature and soil moisture interaction drove nitrous oxide emission.

2.3 Introduction

Ammonia (NH_3) emissions from agriculture contribute to air pollution (Erisman et al., 2008), threaten nitrogen (N) sensitive ecosystems, and can contribute to soil and water acidification (Guthrie et al., 2018). Agricultural sources account for 80–95% of global NH_3 emissions with c. 20% attributed to fertiliser use (Skorupka and Nosalewicz, 2021). Likewise, emissions of nitrous oxide (N_2O), which has a 100-year global warming potential 273 times that of carbon dioxide (IPCC, 2021) and also depletes tropospheric ozone (Sutton et al., 2013), are mainly from N fertilisers and manure applied to crops (IPCC, 2021).

Urea is the most used N fertiliser globally, and in 2020 it represented c. 50% of total synthetic N fertiliser use (IFASTAT, 2022). The dominance of urea is due to its high N content (46% N), low production and storage costs, and flexibility as a soil or foliar application (Cantarella et al., 2008; Matczuk and Siczek, 2021; Silva et al., 2017; Wang

et al., 2020). However, it can be subject to high losses via NH_3 volatilisation following surface application (Matczuk and Siczek, 2021) with reported losses of up to 40% of the applied N (Cantarella et al., 2018; Folina et al., 2021). Urea hydrolysis results in a sharp increase in pH around the vicinity of urea granules, causing a shift from NH_4^+ towards volatile NH_3 (Harrison and Webb, 2001). The rate of hydrolysis and NH_3 loss depends on various factors including soil pH and soil pH and NH_4^+ buffering by reaction with the soil solid, as well as soil temperature and moisture (Cameron et al., 2013; Matczuk and Siczek, 2021). These factors affect urease enzyme activity (Matczuk and Siczek, 2021) and rates of movement of urea and ammoniacal-N and pH changes through the soil away from urea application zone (Rachhpal-Singh and Nye, 1986).

One strategy to reduce NH_3 emissions from urea is the co-application of urease inhibitors (Matczuk and Siczek, 2021). Urease inhibitors temporarily inhibit soil urease activity, retarding urea hydrolysis and minimising the pH increase around urea granules, thereby reducing subsequent NH_3 volatilisation from surface applied urea prior to its movement deeper into the soil by diffusion and mass flow. Globally, reductions in NH_3 emission from surface applied urea of >50% by UI have been reported across different soils and environmental conditions in both field and laboratory studies (Abalos et al., 2014; Li et al., 2018; Sha et al., 2020). The effect of UI on N_2O emission from urea is uncertain. Several studies found decreased N_2O emission when UI was added with surface-applied urea (Saggar et al., 2013; Sanz-Cobena et al., 2016), whereas others observed an increase (Drury et al., 2017; Martins et al., 2017), or no significant change (Carswell et al., 2019; Wallace et al., 2018; Wang et al., 2020). Nitrous oxide emission is predominantly driven by the rate of N input (Qin et al., 2021) and may be non-linearly related to rates of nitrification and denitrification (Lin and Hernandez-Ramirez, 2021).

The stability and effectiveness of UI can be affected by soil properties (e.g., texture, microbial activity, pH) and temperature and moisture conditions (Folina et al., 2021). Watson et al. (1994) evaluated the UI NBPT (N-(n-butyl) thiophosphoric triamide) across 16 soil types and found it to be more effective in soils prone to NH_3 volatilisation, with high pH and low organic matter content. Similarly, Suter et al. (2011) reported that, under wheat or pasture, NBPT was more effective in alkaline than acidic soils, in soils with low urease activity, and at lower (5–15°C) compared with higher (25°C) soil temperatures.

The half-life of NBPT is shorter at high temperatures, both in storage (Watson et al., 2008) and in soil (Cantarella et al., 2018).

Under current trajectories, mean global air temperatures may increase by up to 4°C by 2100 as a result of human induced climate change (IPCC, 2021). Hotter and drier summers and more extreme weather events are predicted for Europe and the UK (Met office, 2023). To date, few studies have evaluated the effectiveness of UI in reducing NH₃ emission from surface applied urea at temperatures >25°C and soil moisture below 40% water filled pore space (WFPS). There is insufficient evidence to make recommendations on the use of UI in regions experiencing temperatures up to 40°C as well as under agricultural and ecological drought conditions. The use of UI under such conditions may be increasingly important, as agriculture intensifies around the globe to feed the growing world population (Global Food Security, 2023).

The objective of this study was, therefore, to assess the influence of higher temperatures across a range of soil moisture conditions on the effectiveness of UI in reducing NH₃ volatilisation from surface-applied urea, and the impact on N₂O emissions.

2.4 Materials and Methods

2.4.1 Soil preparation and properties

Soil was obtained from a field under grass at Rothamsted Research – North Wyke in the southwest of England (45.294 N, 4.8096 W). The soil is Crediton series, a very stony, loamy, typical brown earth (Dystric Cambisol, FAO classification) derived from Permian and Carboniferous reddish breccia (National Soil Resources Institute, 2021). The field had received 145 kg ha⁻¹ of NPK (25–5–5) fertiliser in the year it was sampled (2020). The average annual rainfall at North Wyke is 1042 mm and mean annual soil temperature is 12°C. Representative soil samples were collected from three separate locations within the field to a depth of 10 cm, with approximately 110 kg (dry weight basis) collected in total. The three soil samples formed the replicates used throughout the incubation experiment. After collection, the soil was sieved (< 2 mm) and air dried to a constant weight. The main soil characteristics are given in Table 2-1.

Table 2-1: Main soil properties

Property	Value
Sand (%)	40.8 ± 1.35
Silt (%)	35.6 ± 0.82
Clay (%)	23.6 ± 0.59
pH (1:2.5 H ₂ O)	6.2 ± 0.02
Cation exchange capacity (meq kg ⁻¹ DW)	93.7 ± 3.19
Total C (g C kg ⁻¹ DW soil)	19.9 ± 0.5
Total N (g N kg ⁻¹ DW soil)	2.1 ± 0.04
Extractable nitrate + nitrite (mg N kg ⁻¹ DW soil)	11.4 ± 0.55
Extractable ammonium (mg N kg ⁻¹ DW soil)	7.8 ± 0.18
Extractable urea (mg N kg ⁻¹ DW soil)	0.4 ± 0.17
Urease activity (ug N g ⁻¹ h ⁻¹)	246.8 ± 34.15
Olsen P (mg P kg ⁻¹ DW soil)	2.4 ± 0.07

DW is dry weight, DI is deionized water and data are means ± standard error with ($n = 3$ except urease activity $n = 9$).

Particle size distribution was measured using the method of Kettler et al. (2001). Soil pH at a soil:water ratio of 1:2.5 (w:v) was measured with a Jenway combination electrode and Model 3320 pH meter, cation exchange capacity by the cobalt hexamine method (ISO 23470, 2007), and total N and C by dry combustion (LECO TruMac Combustion analyser). Extractable soil ammonium and total oxidised N (nitrate plus nitrite) were determined by shaking 5 g soil in 50 cm³ 2 M KCl with 50 mg L⁻¹ phenylmercuric acetate (KCl/PMA) on an orbital shaker (150 rev min⁻¹, 60 min), filtering (Sartorius grade 292 filter paper) and photometric analysis (Aquakem 250, Thermo Scientific, UK). Soil urea-N was analysed in the 2 M KCl/PMA extract as described by Greenan et al. (1995). Soil urease activity was measured after incubating 1 g of moist soil with 1.5 ml of 79.9 mM urea solution at 37°C for two hours, followed by extraction of NH₄-N in a mixture of 2 M KCl and 1 M HCl (KCl/HCl; Kandeler et al., 1999). Plant available P was measured by extraction with NaHCO₃ (Olsen et al., 1954) and colorimetry (Murphy and Riley, 1962).

2.4.2 Experimental design

The experiment consisted of four N fertiliser treatments: urea (U; Diamond Fertilisers, Lincolnshire, UK); urea with the urease inhibitor NBPT (treatment henceforth is US; Origin SUSTAIN®, Hertfordshire, UK) at 600 ppm; urea with two urease inhibitors (treatment henceforth is UL NBPT and N(n-propyl)-thiophosphoric triamide (NPPT) ; N-Shield® Diamond Fertilisers, Lincolnshire, UK) at 410 ppm; and a zero N control (C). The treatments were evaluated at three temperatures (15, 25 and 35°C) and three soil moisture contents (20, 40 and 60% WFPS) to capture typical and extreme high temperatures and typical and extreme low soil moisture conditions. Soil was packed into columns (10 cm depth, 10.3 cm diameter) at a bulk density of 1.1 g cm⁻³. Air-dried soil was pre-wetted with deionised (DI) water to just below the desired WFPS before packing and compressing the soil in 2.5 cm layers, to a depth of 10 cm. After adding each layer of soil, sufficient DI water was added to bring that layer to the desired WFPS. The volume of DI water (V , cm³) needed to achieve the desired WFPS ($WFPS_{desired}$, %) was calculated according to:

$$V = \frac{WFPS_{desired}}{100} \times V_{core} \left(1 - \frac{\rho_b}{\rho_p} \right) \quad (2-1)$$

where V_{core} is the core volume (cm³), ρ_b is bulk density (g cm⁻³) and ρ_p is particle density (g cm⁻³). To maintain soil WFPS over the incubation period, soil columns were watered to their specific weight every 48 h.

The experiment was conducted as a split plot design with a factorial treatment structure. There were several runs in a controlled environment room to allow for different combinations of temperature and moisture and there were three blocks within each run. Temperature and moisture were applied to blocks within runs and treatments were applied to chambers within each block. The soil incubation post fertilisation was conducted over thirty days after initially equilibrating the chambers at the designated temperature for seven days. After temperature equilibration, N fertiliser treatments were surface applied to the soil at a rate of 70 kg N ha⁻¹ and gas sampling was initiated. The rate of N applied

was based on the recommended N rate (on average) for cereal production in some Sub-Saharan Africa countries (CNRA, 2021).

Additional soil columns (depth of 10 cm, and internal diameter of 4.3 cm) were prepared and equilibrated for seven days at the experimental temperature and moisture settings to allow destructive soil sampling at t_0 to be carried out. These cores were covered with Parafilm and punctured to allow gas exchange. At the end of the equilibration period, these cores were sub-sampled at 0–1.25, 1.25–2.5, 2.5–5 and 5–10 cm depth and extracted with KCl/PMA or KCl/HCl for determination of NH_4 , total oxidised N and urea-N. Urease activity was also determined on a bulked depth sample. Total dissolved N was extracted from 10 g of the pre- and post-incubated soils using 50 ml 0.5 M K_2SO_4 solution and analysed with a TOC/TN analyser (Shimadzu UK Ltd, Milton Keynes, UK).

2.4.3 Gas flux measurements

Cumulative NH_3 emissions were determined from each chamber using an air throughflow system based on that of Misselbrook et al. (2005). Air was passed through 80 ml of 0.02 M H_3PO_4 prior to entering the soil chamber headspace (to remove any background NH_3 in the air) and through another 80 ml of 0.02 M H_3PO_4 after passing through the chamber headspace to capture volatilised NH_3 . The chamber system was tested prior to experimentation, and an average NH_3 -N recovery of 98% was measured (across all chambers and two runs). The air flow system for NH_3 measurement ran continuously through the incubation, except whilst acid traps were changed or when sampling for N_2O (which typically lasted 2.5 h). The inlet and outlet acid traps of each chamber were changed daily for the first 14 days of the experiment, and 3 times weekly after that. The outlet acid trap solutions were transferred and made up to 100 ml volume in volumetric flasks, then subsampled and stored at 4°C in the dark before analysis of NH_4 -N using automated colorimetry (Aquakem 250 discrete photometric analyser; Thermo Scientific, UK).

Nitrous oxide fluxes were measured using a closed chamber approach, according to de Klein et al. (2012). Air (30 ml) was sampled from the chamber headspace after sealing the lid, at 0, 20, 40 and 60 min and transferred to 22 ml vials. To ensure equilibrium of pressure within the chambers, 30 ml of ambient air was added to the chamber head space

following each sample removal through a side air entry point. This introduction of ambient air was accounted for in subsequent calculations. Gas sampling started one day prior to N fertiliser application. As for NH₃, N₂O sampling was conducted daily for the first 14 days following N fertiliser application and 3 times a week after that. The N₂O concentrations in the chamber headspace were measured using a Perkin Elmer Clarus 580 Gas Chromatograph, fitted with an electron capture detector, and a flame ionisation detector housing a methanizer.

2.4.4 Data analyses

The rate of NH₃ emission (F , g N m⁻² h⁻¹) was estimated according to (Misselbrook et al., 2005):

$$F = \frac{XV}{At} \quad (2-2)$$

where X is the ammoniacal-N concentration measured from the outlet acid trap solution (g l⁻¹), V is the volume of this solution (l), A is the soil surface area (m²), and t is the duration of the sampling period (hours). The flux during periods when NH₃ sampling was paused was calculated as the average of the flux in the period immediately before and immediately after the air flow was switched off. Total NH₃ emission (g NH₃-N) for the sampling period was determined as XV . The cumulative NH₃ emission throughout the incubation (following fertiliser application) was calculated by summing emissions for each sampling period. The percentage of the urea-N applied to each chamber lost as NH₃-N was calculated according:

$$\% \text{ NH}_3 \text{ loss} = \frac{\text{NH}_3 \text{ emitted}_{\text{net}}}{\text{applied N}} \times 100 \quad (2-3)$$

where NH₃ emitted_{net} (g N m⁻²) is the emission value obtained after subtracting the control treatment (no urea-N applied) from the total emission for each sampling period, and applied N is the amount of N applied (g) as urea.

Nitrous oxide fluxes were calculated from the linear increase in N₂O concentrations between $t = 0$ and $t = 60$ min (de Klein et al., 2012), as follows:

$$F = H \frac{dC}{dt} \quad (2-4)$$

where F is N_2O flux ($\mu\text{g N}_2\text{O-N m}^{-2} \text{ s}^{-1}$), H is the ratio of the internal chamber volume (m^3) to soil surface area (m^2), C is the N_2O concentration in the chamber (mg N l^{-1}), and t is time of sampling. The designation dC/dt is used to represent the time rate of change in C (slope of the linear regression). Cumulative N_2O emissions between two sampling events were calculated as the product of the mean flux rate and the time interval between the measurements. These were then summed to derive cumulative gas loss for the duration of the experiment.

Treatment effects on the cumulative NH_3 and N_2O emissions, as well as the temperature and moisture effects on the emission reduction potential of the urease inhibitors were evaluated using a linear mixed model (REML) with block ((experiment.replicate)/chamber/sampling number) as random effect and fixed term as (temperature*moisture*fertiliser treatment*day). Pairwise comparisons of the data were made using the least significant difference test, with differences between treatments considered significant where $p < 0.05$. All data required transformation (square root) to meet model assumptions of data normality. Statistical analyses were carried out using GenStat (21st edition).

2.5 Results

2.5.1 Temperature and soil moisture effects on daily ammonia flux

Daily NH_3 flux was significantly impacted by fertiliser treatment, temperature, soil moisture and their interaction (Tables 2-2 and 2-3). Flux from the control treatment was negligible at all temperatures and soil moisture conditions. Average daily flux (across the 30-d measurement) for U increased with increasing temperature and decreased with increasing soil moisture (Figure 2-1). The average daily flux for U was generally greatest at 35°C ($0.10 \text{ g NH}_3\text{-N m}^{-2} \text{ d}^{-1}$) followed by 25°C and 15°C under the same WFPS, except at 20% WFPS where the average daily flux at 15 and 25°C was not statistically different (0.07 and $0.08 \text{ g NH}_3\text{-N m}^{-2} \text{ d}^{-1}$, respectively). However, at 20% WFPS, the peak emissions at 25 and 35°C were not significantly different, but were 32% greater, on average, than that at 15°C . At 40% WFPS, average daily flux for U was 0.03 , 0.05 and $0.07 \text{ g NH}_3\text{-N m}^{-2} \text{ d}^{-1}$ at 15, 25 and 35°C , respectively, with peak emission being similar at 35°C and 25°C , 48% greater than that at 15°C . At 60% WFPS, average daily flux was 0.01 , 0.03 and $0.04 \text{ g NH}_3\text{-N m}^{-2} \text{ d}^{-1}$ at 15, 25 and 35°C , respectively, with peak emissions

at 25 and 35°C greater by 75 and 65%, respectively, compared to 15°C. Most emissions from U occurred during the first 14 days after N addition, with emission peaks occurring on day 1 at 25 and 35°C and on day 2 at 15°C (Figure 2-1).

Table 2-2: Daily and cumulative NH₃-N and N₂O-N emissions according to linear mixed effects model in response to temperature, moisture, and nitrogen source.

	NH ₃ flux (g N m ⁻² d ⁻¹)	Cumulative NH ₃ loss (g N m ⁻²)	N ₂ O flux (g N m ⁻² d ⁻¹)	Cumulative N ₂ O loss (g N m ⁻²)
Temperature (°C)	LSD (0.0035)	LSD (0.0564)	LSD (0.0034)	LSD (0.0655)
15	0.4733 ± 0.0011 [0.0158]	0.4497 ± 0.0190 [0.3271]	0.4676 ± 0.0011 [0.0088]	0.4287 ± 0.0220 [0.1701]
25	0.4786 ± 0.0011 [0.0219]	0.5987 ± 0.0190 [0.5028]	0.4684 ± 0.0011 [0.0095]	0.4540 ± 0.0223 [0.1901]
35	0.4933 ± 0.0012 [0.0359]	0.8476 ± 0.0190 [0.9253]	0.4682 ± 0.0012 [0.0094]	0.4153 ± 0.0219 [0.1604]
Moisture (% WFPS)	LSD (0.0034)	LSD (0.0564)	LSD (0.0034)	LSD (0.0655)
20	0.0439 ± 0.0034 [0.0439]	0.8544 ± 0.0190 [0.9776]	0.4675 ± 0.0012 [0.0087]	0.4156 ± 0.0223 [0.1531]
40	0.0221 ± 0.0018 [0.0221]	0.5787 ± 0.0190 [0.4793]	0.4706 ± 0.0011 [0.0116]	0.4693 ± 0.0220 [0.2041]
60	0.0121 ± 0.0011 [0.0121]	0.4629 ± 0.0190 [0.2983]	0.4665 ± 0.0011 [0.0077]	0.4131 ± 0.0219 [0.1634]
Fertiliser	LSD (0.0031)	LSD (0.0566)	LSD (0.0030)	LSD (0.0544)
C	0.4593 ± 0.0012 [0.0010]	0.1431 ± 0.0205 [0.0228]	0.4662 ± 0.0011 [0.0075]	0.3853 ± 0.0208 [0.1418]
US	0.4811 ± 0.0012 [0.0229]	0.7021 ± 0.0205 [0.6105]	0.4692 ± 0.0011 [0.0104]	0.4501 ± 0.0208 [0.1919]
UL	0.4820 ± 0.0012 [0.0240]	0.6482 ± 0.0205 [0.5566]	0.4681 ± 0.0012 [0.0092]	0.4404 ± 0.0213 [0.1742]
U	0.5017 ± 0.0012 [0.0474]	1.0347 ± 0.0205 [1.1504]	0.4687 ± 0.0011 [0.0098]	0.4549 ± 0.0208 [0.1862]
<i>Significance of fixed effects</i>				
Temperature	<0.001	<0.001	0.919	0.517
Moisture	<0.001	<0.001	0.058	0.148
Fertiliser	<0.001	<0.001	0.258	0.051
<i>Significance of interaction effects</i>				

Temperature × moisture	<0.001	0.007	0.055	0.003
Temperature × fertiliser	<0.001	<0.001	0.079	0.024
Moisture × fertiliser	<0.001	<0.001	0.173	0.071
Temperature × fertiliser × moisture	<0.001	0.187	0.983	0.911

Values are predicted mean values $\pm$ standard error ($n = 3$) from REML analysis, with real means shown within brackets. The least significant difference values were determined at $p < 0.05$.

Table 2-3: Mean daily NH₃-N emissions under varying temperature, moisture, and nitrogen sources.

Fertiliser	Daily NH ₃ Flux (g N m ⁻² d ⁻¹)			
	Moisture (% WFPS)	20	40	60
	Temperature (°C)			
C	15	0.4586 ± 0.0034 [0.0003]	0.4588 ± 0.0034 [0.0005]	0.4588 ± 0.0034 [0.0005]
	25	0.4596 ± 0.0034 [0.0013]	0.4594 ± 0.0034 [0.0011]	0.4596 ± 0.0033 [0.0013]
	35	0.4595 ± 0.0040 [0.0012]	0.4603 ± 0.0034 [0.0019]	0.4593 ± 0.0033 [0.0010]
US	15	0.4953 ± 0.0034 [0.0375]	0.4633 ± 0.0034 [0.0047]	0.4603 ± 0.0034 [0.0019]
	25	0.4983 ± 0.0034 [0.0407]	0.4688 ± 0.0034 [0.0099]	0.4691 ± 0.0033 [0.0104]
	35	0.5555 ± 0.0041 [0.1062]	0.4955 ± 0.0034 [0.0359]	0.4800 ± 0.0033 [0.0209]
UL	15	0.4929 ± 0.0034 [0.0345]	0.4627 ± 0.0034 [0.0042]	0.4600 ± 0.0034 [0.0017]
	25	0.4834 ± 0.0034 [0.0255]	0.4696 ± 0.0034 [0.0107]	0.4669 ± 0.0033 [0.0082]
	35	0.5726 ± 0.0038 [0.1260]	0.5057 ± 0.0034 [0.0472]	0.4771 ± 0.0033 [0.0179]
U	15	0.5233 ± 0.0034 [0.0747]	0.4889 ± 0.0034 [0.0314]	0.4690 ± 0.0034 [0.0104]
	25	0.5238 ± 0.0034 [0.0780]	0.5007 ± 0.0034 [0.0466]	0.4880 ± 0.0033 [0.0333]
	35	0.5328 ± 0.0039 [0.0767]	0.5205 ± 0.0034 [0.0645]	0.4942 ± 0.0033 [0.0363]

Values are predicted mean values ± standard error (n = 3) from REML analysis, with real means shown within brackets. The least significant difference value was 0.0097 at p < 0.05.

The inclusion of UI with urea significantly ($p < 0.001$) reduced the average daily NH_3 flux compared to untreated urea and peak emission was generally reduced and delayed (Figure 2-1). The UI treatments, US and UL did not differ significantly at all temperatures and soil moistures. The reduction in peak emission for UI treatments, compared with U, typically decreased with increasing temperature and with decreasing soil moisture (Figure 2-1). At 20% WFPS, UI reduced peak emission relative to U by 64, 72, and 46% at 15°C, 25°C and 35°C, respectively; at 40% WFPS, respective reductions were 86, 86, and 58%, and at 60% WFPS, respective reductions were 91, 80 and 60%. The average daily NH_3 flux for the UI treatments (US and UL treatments were not significantly different) was 0.04, 0.04 and 0.08 g $\text{NH}_3\text{-N m}^{-2} \text{ d}^{-1}$ at 15, 25 and 35°C, respectively at 20% WFPS, 0.004, 0.01 and 0.05 g $\text{NH}_3\text{-N m}^{-2} \text{ d}^{-1}$, respectively at 40% WFPS, and 0.002, 0.01 and 0.02 g $\text{NH}_3\text{-N m}^{-2} \text{ d}^{-1}$, respectively at 60% WFPS. Inclusion of UI with U delayed the NH_3 emission peak relative to U at all temperatures and soil moistures, with the delay decreasing as temperature increased, being 4 days at 15°C, 3 days at 25°C, and 2 days at 35°C. At 35°C, across all soil moistures and for U and UI treatments, NH_3 emissions persisted for up to 20–25 days after treatment application, whereas at 15 and 25°C NH_3 emissions returned to baseline (that of the C treatment) by 14 days. Prolonged emission up to 19 days was also noticeable at 15°C and 20% WFPS for UI treatments.

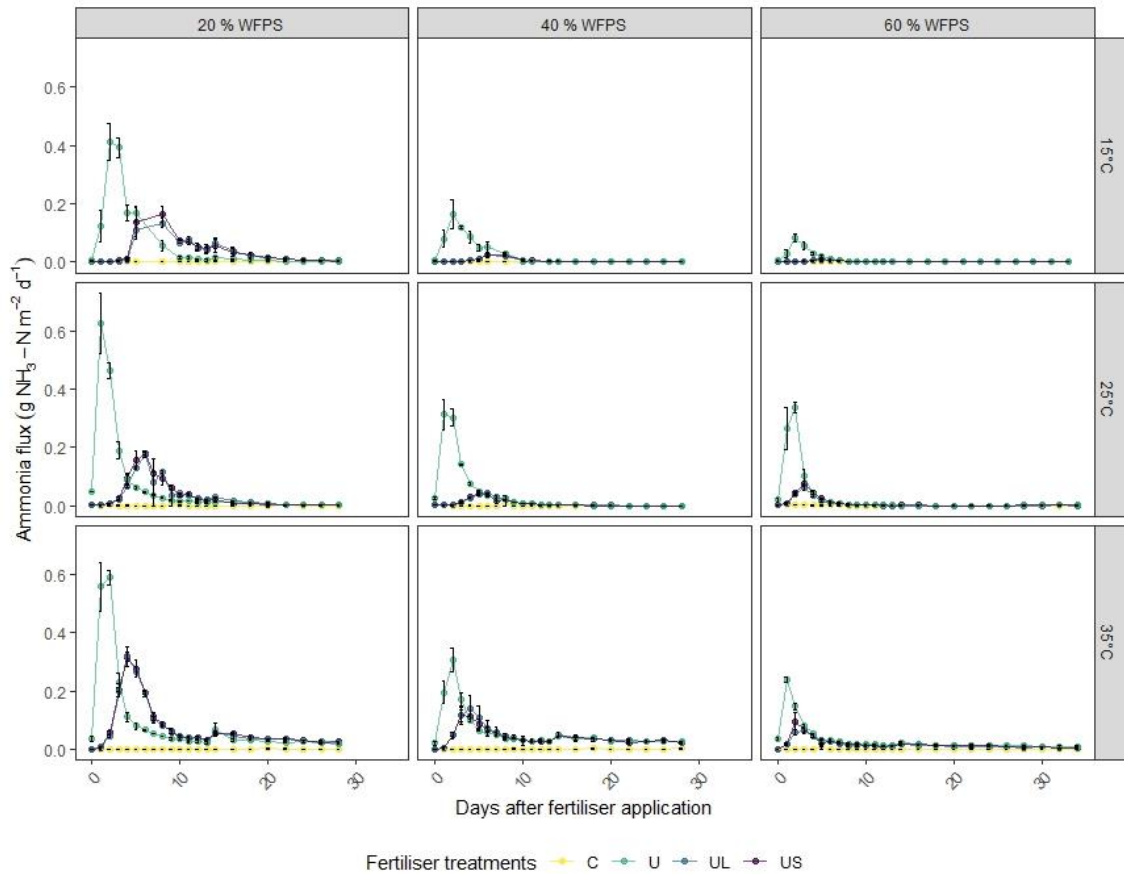


Figure 2-1: Ammonia flux (g NH₃-N m⁻² d⁻¹) over thirty days following treatment application, of the control (C), urea (U) and U with urease inhibitors (UL and US) at 15, 25 and 35°C and at 20, 40 and 60% water filled pore space (WFPS). Datapoints represent mean values and vertical error bars depict standard error of mean ($n = 3$).

2.5.2 Temperature and soil moisture impact on cumulative ammonia emissions

Cumulative NH₃ emissions were significantly affected by temperature, soil moisture and fertiliser treatment (Table 2-2), and all two-way interactions between these three factors were also significant. Cumulative NH₃ emission was negligible from the control treatment but increased with increasing temperature and decreasing soil WFPS for all fertiliser treatments (Figure 2-2). The greatest cumulative NH₃ emissions were from U at 20% WFPS, at 1.42, 1.72 and 2.19 g NH₃-N m⁻² at 15, 25 and 35°C, respectively, accounting for 20, 24 and 31%, respectively, of the applied urea-N. At 40% WFPS, cumulative NH₃ emissions were 0.60, 1.02 and 1.42 g NH₃-N m⁻² at 15, 25 and 35°C, respectively, accounting for 8, 14 and 19%, respectively, of the applied urea-N. At 60% WFPS,

cumulative NH_3 emissions were 0.25, 0.83 and 0.91 $\text{g NH}_3\text{-N m}^{-2}$ at 15, 25 and 35°C, respectively, accounting for 3, 11 and 13%, respectively, of the applied urea-N.

At all temperatures and soil moistures, cumulative NH_3 emissions were significantly greater from U relative to the UI treatments (Figure 2-2), which were equally effective in reducing the emission. However, the efficacy of the UI in reducing emissions decreased with increasing temperature and with declining soil moisture. The inclusion of UI with urea (averaged across both UI treatments) reduced cumulative NH_3 losses relative to urea alone by 87, 86 and 52% at 15°C, by 75, 79 and 50% at 25°C, and by 48, 33, 22% at 35°C for 20, 40 and 60% WFPS, respectively.

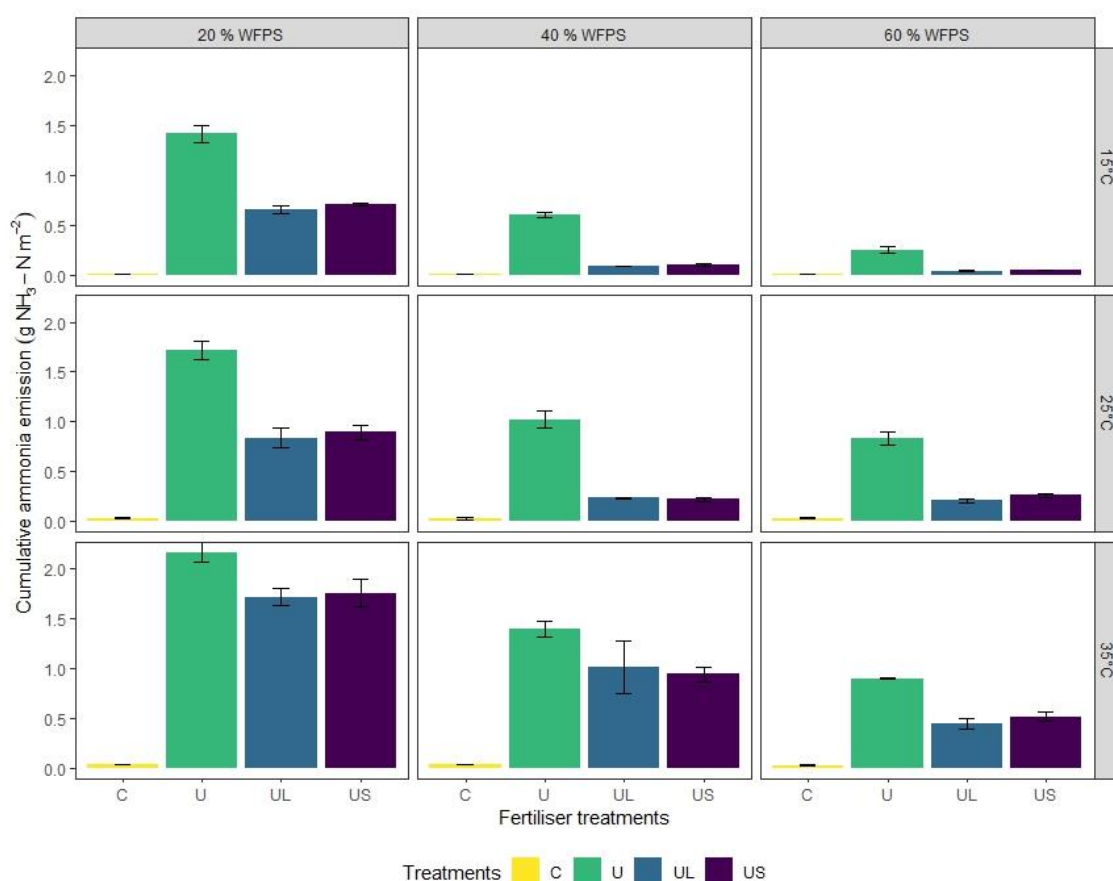


Figure 2-2: Cumulative ammonia emissions ($\text{g NH}_3\text{-N m}^{-2}$) over the thirty-day incubation period, after application of the control (C), urea (U) and urea with urease inhibitor (UL and US) treatments at 15, 25, 35°C and 20, 40 and 60% water filled pore space (WFPS). Datapoints represent mean values and vertical bars depict standard error of the mean ($n = 3$).

2.5.3 Temperature and moisture effect on nitrous oxide emission from N treatments

There was no significant effect of fertiliser treatment or temperature on average daily N_2O flux (Table 2-2). However, there was a significant effect of moisture and the interaction between temperature and moisture. Average daily N_2O -N flux at 40% WFPS was significantly greater than that at 20 or 60% WFPS, which were not significantly different from each other. At the lowest incubation temperature of 15°C, average daily N_2O -N flux was significantly greater at 40% WFPS than at 20 and 60% WFPS, whereas at 25°C, there was no significant difference in the average daily N_2O -N flux at 40 and 20% WFPS but both were greater than that at 60% WFPS (Figure 2-3). At the highest incubation temperature of 35°C, there was no significant effect of soil moisture.

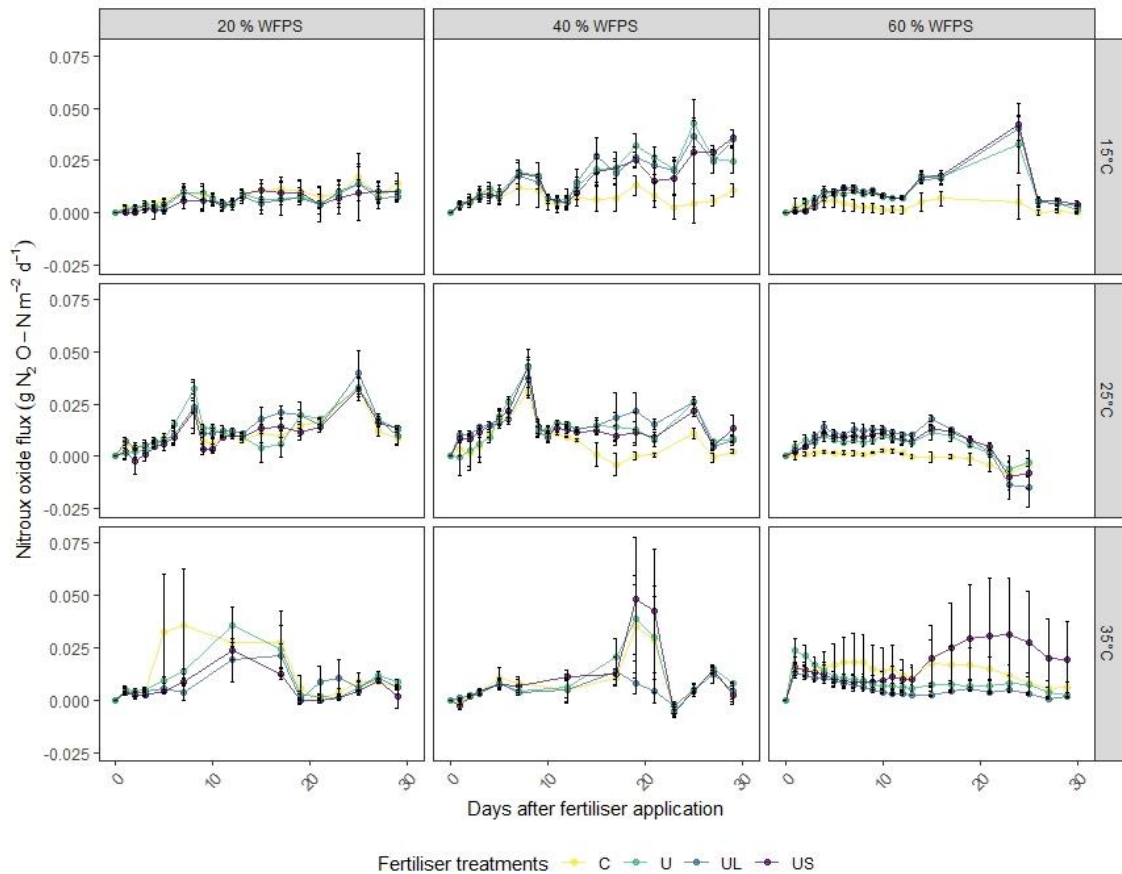


Figure 2-3: Nitrous oxide flux ($\text{g N}_2\text{O-N m}^{-2} \text{d}^{-1}$) over thirty days following treatment application, of the control (C), urea (U) and U with urease inhibitors (UL and US) at 15, 25 and 35°C and at 20, 40 and 60% water filled pore space (WFPS). Datapoints represent mean values and vertical error bars depict standard error of mean ($n = 3$).

Cumulative N₂O-N emission was significantly affected by fertiliser treatment and the interactions between temperature and fertiliser treatment and temperature and moisture (Table 2-2). The C treatment had significantly less cumulative N₂O-N losses than US, UL and U which were not significantly different from each other. Additionally, the interaction between fertiliser treatment and temperature was predominantly related to the differences between the C treatment and the other fertiliser treatments (Figure 2-4). Across all fertiliser N treatments, the cumulative N₂O emission at 15°C was greater at 40% WFPS compared to 20% WFPS, however, both were not significantly different from cumulative N₂O emission at 60% WFPS. At 25°C, cumulative N₂O-N emission was significantly greater at 40 and 20% WFPS relative to 60% WFPS. At 35°C, cumulative emission at 60% WFPS was significantly greater than that at 40 and 20% WFPS. Averaged across the U, US and UL treatments, at 15°C net cumulative N₂O emission accounted for 0.00, 2.38 and 1.89 % of the applied N, at 20, 40 and 60% WFPS, respectively (Figure 2-4). At 25°C cumulative net emission accounted for 0.53, 1.76, and 1.80% of applied N, and 0.00, 0.32, and 0.41% of applied N at 35°C, at 20, 40 and 60% WFPS, respectively.

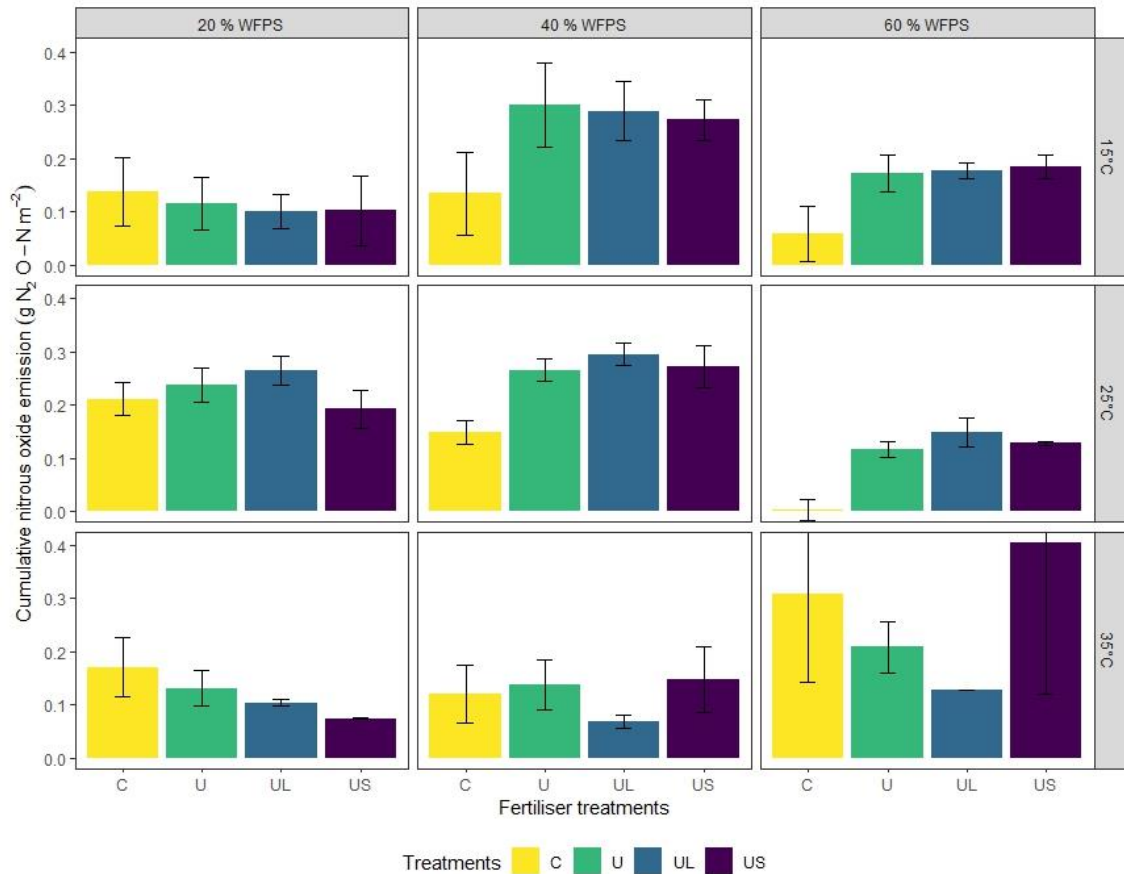


Figure 2-4: Cumulative nitrous oxide emissions (g N₂O-N m⁻²) over the thirty-day incubation period for the control (C), urea (U) and urea with urease inhibitor (UL and US) treatments at 15, 25, 35°C and 20, 40 and 60% water filled pore space (WFPS). Datapoints represent mean values and vertical bars depict standard error of the mean ($n = 3$).

2.5.4 Soil urease activity, ammonium, nitrate, and total N

Soil urease enzyme activity after 7 days preincubation was significantly affected by the interaction between temperature and soil moisture ($p < 0.030$). Urease activity was generally significantly higher at 35°C (286 and 178 $\mu\text{g N g}^{-1} \text{h}^{-1}$) and 25°C (201 and 102 $\mu\text{g N g}^{-1} \text{h}^{-1}$) under 20 and 40% WFPS, respectively, compared to 60% WFPS where there was no significant difference between temperature. Moisture effect on urease activity was not consistent and varied at each temperature.

Following incubation, soil total dissolved N content was greater for the N fertiliser treatments, which did not differ significantly, than the C treatment across all temperatures and soil moistures (Figure 2-5; soil NH₄-N and NO₃-N concentrations are presented in supplementary Figures B-1 and B-2). The N concentrations decreased with soil depth,

and at 10 cm there were no significant differences between the C and other N treatments for any N form. Greatest retention of applied N was at 15°C and 20% WFPS, and least retention of N at 15°C and 60% WFPS and 35°C and 40% WFPS.

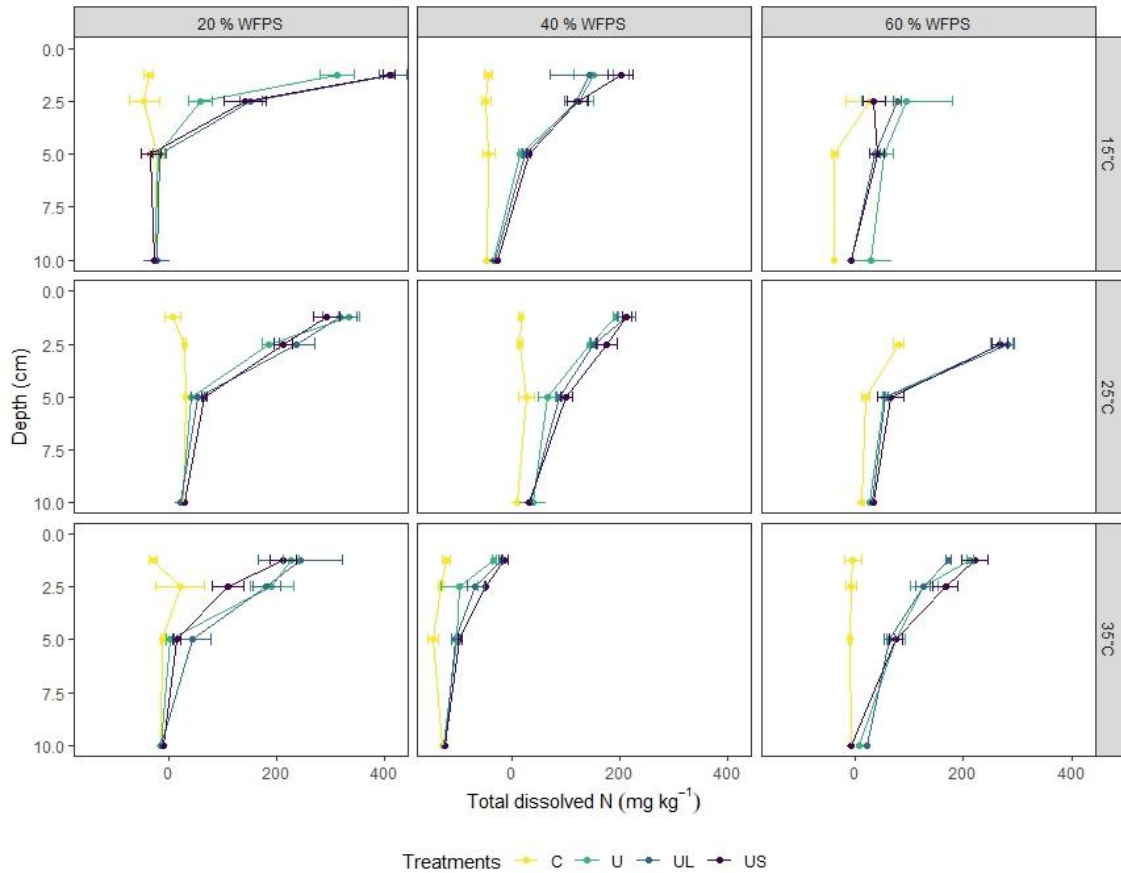
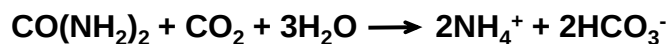


Figure 2-5: Soil total dissolved N concentration following incubation for the control (C), urea (U) and urea with urease inhibitor (UL and US) treatments at 15, 25, 35°C and 20, 40 and 60% water filled pore space (WFPS). Datapoints represent mean values and vertical bars depict standard error of the mean ($n = 3$).

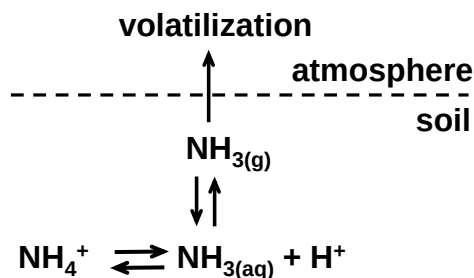
2.6 Discussion

2.6.1 Temperature and moisture effects on NH_3 loss from urea

The following processes need to be considered to understand the combined effects of temperature and moisture on NH_3 loss from urea applied to soil (for a mechanistic model, see Rachhpal-Singh and Nye, 1986). Urea dissolves rapidly in the soil solution and is then hydrolysed to ammonium (NH_4^+) and bicarbonate (HCO_3^-) ions in the following reaction catalysed by urease:



The HCO_3^- ions so formed tend to raise the soil pH, and this favours conversion of NH_4^+ to NH_3 , which is volatile:



‘Volatilisation’ is the transfer of NH_3 gas from the soil surface to the atmosphere above. The reaction scheme above shows that during volatilisation, $\text{NH}_4\text{-N}$ (i.e., $\text{NH}_4^+ + \text{NH}_3$) at the soil surface is depleted, and H^+ ions will accumulate tending to decrease the soil pH, but they will be neutralised by diffusion of HCO_3^- ions. The overall rate of volatilisation therefore depends on the following linked rate processes, each of which is to some extent influenced by temperature and moisture:

1. movement of dissolved urea into the soil and its simultaneous hydrolysis;
2. movement of $\text{NH}_4\text{-N}$ away from the zone of urea hydrolysis and towards the depletion zone at the soil surface, with simultaneous diffusion of gaseous NH_3 in the soil air and its transfer across the soil-atmosphere boundary;
3. movement of acids and bases (principally $\text{H}_3\text{O}^+\text{-H}_2\text{O}$ and $\text{H}_2\text{CO}_3\text{-HCO}_3^-$) to and from the zones of urea hydrolysis and $\text{NH}_4\text{-N}$ depletion.

A decrease in soil moisture will tend to decrease rates of diffusion of dissolved urea, $\text{NH}_4\text{-N}$ and acids and bases through the soil, but it will also increase the soil air space, and therefore the gaseous diffusion of NH_3 to the soil surface. We have found increased volatilisation with decreased soil moisture content in our experiments, suggesting the latter effect is the more important. Likewise, Rachhpal-Singh and Nye (1986) found by modelling that volatilisation increased with decreases in soil moisture content below field capacity. In our experimental system the soil moisture content was constant. But under evaporating conditions, mass flow of dissolved reactants with water towards the soil

surface will increase rates of volatilisation, and vice versa for infiltration (Freney et al., 1981; Kirk and Nye, 1991).

Up to some temperature optimum, urease activity and hence urea hydrolysis will increase with temperature (Suter et al., 2011). Rates of diffusion of NH_3 gas and the dissolved reactants will tend to increase with soil temperature, as will NH_3 transfer across the soil-atmosphere boundary (Kirk and Nye, 1991). The solubility of NH_3 gas decreases with increasing temperature, and the dissociation of NH_4^+ increases, both of which will favour increased volatilisation (Kirk and Nye, 1991).

In our incubation study, we observed the lowest emissions from the soils with the highest soil moisture (60% WFPS) and the highest emissions under the driest soil (20% WFPS). Similarly, Yerokun, (1997) reported higher NH_3 volatilisation when soil moisture was maintained at 25% field capacity, compared to soil moisture adjusted to 100% field capacity. McGarry et al. (1987) also reported NH_3 losses to increase with decreasing soil moisture content. Because of the hygroscopic nature of urea, its granules readily dissolve on the soil surface, even where soil moisture is low, allowing hydrolysis of urea and therefore NH_3 volatilisation (Dari et al., 2019).

As observed by Li et al. (2017) in a sub-humid continental climate in China, initial conditions of light precipitation (2–4 mm) with high relative humidity (60–78%) were favourable for NH_3 volatilisation. Low rainfall and high air humidity can provide sufficient moisture for urea hydrolysis and the desorption of ammoniacal-N from the soil but not enough to move urea and $\text{NH}_4^+/\text{NH}_3$ into the soil profile, resulting in substantial NH_3 volatilisation (Craig and Wollum, 1982). However, conditions of low relative air humidity (35–50%) and heavy rainfall (60 mm) after N top dressing can limit NH_3 volatilisation (Li et al., 2017) because urea is displaced deeper into the soil. Under high soil moisture, there is greater solubilisation and diffusion of urea into the soil, thus reducing urea hydrolysis on the soil surface and susceptibility to volatilisation (Carswell et al., 2019; Holcomb et al., 2011). An incorporation of urea to a depth of 5 cm or more can help to minimize NH_3 volatilisation (Fenn and Miyamoto, 1981; Holcomb et al., 2011). Scenarios of wet and drying soil events can also greatly influence NH_3 emissions as reported by Engel et al. (2011), with substantial NH_3 losses (30–44% of the applied N) when urea was applied to a wet or damp soil surface followed by a drying soil due to low

rainfall, but much lower losses (<10% applied N) when the urea was applied to a dry soil surface followed by abundant rainfall in a winter wheat field. These findings illustrate the importance of wetting and drying soil events for NH_3 emissions from urea.

2.6.2 The efficacy of urease inhibitors in reducing ammonia losses as affected by temperature and moisture

The peaks in NH_3 emission were delayed and reduced where urea was treated with UI in this study, as reported elsewhere (e.g., Martins et al., 2017; Tian et al., 2015; Wang et al., 2020). In our study, the inclusion of UI (as US and UL) with urea delayed the emission peaks by 2 to 4 days after fertiliser addition. A delay in peak emission of 1 day through use of NBPT was observed by Wang et al. (2020) for a loamy loess soil (pH 7.2) at 55% WFPS and incubated at 25°C for 30 days, whereas Soares et al. (2012) reported a longer delay of 7–9 days for a Red Latosol (pH 5.9) at 60% WFPS and incubated at 25°C for 22 days. A metanalysis by Silva et al. (2017) showed that inclusion of NBPT delayed peak NH_3 loss by 3.5 days on average across a variety of climatic regions (temperate, cold, tropical), with peak NH_3 emission occurring on average at day 4.8 and 8.3 after surface application of urea and urea treated with NBPT, respectively. In a study by Abalos et al. (2012), UI did not delay the NH_3 peak emission, but the peak from the urea treated with NBPT was reduced by a factor of 2.4 compared with that from urea, and Wang et al. (2020) reported peak NH_3 emission of $0.27 \text{ g NH}_3\text{-N m}^{-2} \text{ d}^{-1}$ for NBPT treated urea, compared with $0.84 \text{ g NH}_3\text{-N m}^{-2} \text{ d}^{-1}$ for urea. Our study is consistent with these findings, as UI reduced peak NH_3 emission by >40% relative to urea alone, across all temperature and moisture conditions.

Application of UI delays urea hydrolysis and slows NH_4^+ release and increase in soil pH, therefore delaying and reducing the peak NH_3 emission rate and enabling urea movement deeper into the soil (Suter et al., 2011; Tasca et al., 2011). The inclusion of NBPT and NBPT/NPPT with urea has been shown to greatly reduce urease activity for up to 15 days after urea application, depending on soil temperature and soil type (Li et al., 2017; Sanz-Cobena et al., 2011).

Most of the $\text{NH}_3\text{-N}$ losses from our experiment happened within the first 14–15 days after N application. Nonetheless, at 35°C, despite the evident delayed and reduced NH_3 emission by UI over U, $\text{NH}_3\text{-N}$ emissions for both U and UI treatments continued for up

to 20 days (particularly at 20 and 40% WFPS). Similar prolonged NH_3 emissions from both urea and urea plus UI was reported by Tian et al. (2015) in a subtropical cotton field, where major losses of NH_3 occurred within 30 days but persisted up to 40 days at high mean temperature of 31.8°C and 14.4–15.1% moisture (w/w). The prolonged emissions in our study could be due in part to higher concentrations of NH_4^+ in the upper soil surface (0–1.25 cm depth) after urea hydrolysis at 35°C and low WFPS (20 and 40%) as observed from soil analysis at the end of experiments. The combined effect of high temperature and low moisture will have favoured volatilisation by upward gaseous diffusion of NH_3 (Siman et al., 2020). Several authors have reported prolonged NH_3 emission from urea in dry soils at various temperatures ($5\text{--}25^\circ\text{C}$), suggesting low moisture was the driving factor and not temperature (Holcomb et al., 2011).

Similar to the effect on peak emission rate, cumulative NH_3 emission over 30 days was significantly lower (by 22–87%) from the UI treatments relative to urea alone, consistent with reductions reported elsewhere (Carswell et al., 2019; Silva et al., 2017; Tian et al., 2015). However, it was evident from our study that the efficacy of the UI decreased with increasing temperature and with decreasing soil moisture. A similar temperature effect, across the range $5\text{--}25^\circ\text{C}$ was reported by Suter et al. (2011) for applications to wheat and pasture soils in south-eastern Australia. Siman et al. (2020) observed only limited efficacy of NBPT (c. 8% emission reduction) at high temperature (45°C) and intermediate soil moisture (around 10% gravimetric water content). At elevated temperature, NBPT undergoes fast degradation, shortening the inhibition effect and leading to greater NH_3 volatilisation (Clay et al., 1990; Engel et al., 2011). In addition, at high temperatures, the lower UI efficacy can be due to the rate of urea hydrolysis exceeding the rate of NBPT conversion into its oxygen analogue NBPTO (N-(n-butyl) phosphoric triamide), which is the direct inhibitor of urease (Oliveira et al., 2014). Our findings show a clear difference in UI efficacy between 25 and 35°C , in contrast to Siman et al. (2020), who suggested that the decomposition of NBPT to NBPTO in soil only occurs at temperatures above 35°C . The increase in soil moisture enhanced UI efficacy and reduced NH_3 volatilization, especially at low temperature in our study. However, NBPT efficacy can be limited at WFPS above 65% (Sanz-Cobena et al., 2012). For instance, in subtropical agroecosystems in Brazil, Ribeiro et al. (2020) found UI had little effect during winter because of significant rainfall after N application.

2.6.3 Effect of soil conditions and urease inhibitors on nitrous oxide emission

In our study, N₂O emissions were affected by temperature and predominantly soil moisture, with greater emissions at 40% WFPS at 15 and 25°C, although the highest N₂O emission at 35°C was at 60% WFPS. Our findings align with those of Wallace et al. (2018) who observed a noticeable increase in N₂O emissions from a neutral-alkaline clay soil after N application when soil moisture was between 40 and 60% WFPS, suggesting nitrification was the main source. Our findings contrast with the observations of Sanz-Cobena et al. (2016), who reported negligible N₂O emission at 40% WFPS and greater emissions at 60 and 80% WFPS. They observed the greatest emission at 60% WFPS, considered as an optimum condition for nitrification, and a threshold for water and aeration controlling soil microbial processes (Menéndez et al., 2012). Sey et al. (2008) measured higher soil respiration from heterotrophic microorganisms at 40% and at 80% WFPS, both corresponding to N₂O peaks, however overall N₂O emissions were greatest at 80% WFPS. Nitrous oxide emissions are predominantly from autotrophic nitrification at lower soil moisture contents and denitrification at moisture contents above 60% WFPS (Bateman and Baggs, 2005). However, denitrification can occur at soil moisture < 60% WFPS due to anaerobic microsites within soil aggregates (Martins et al., 2017; Sey et al., 2008). Overall, our findings suggest that soil moisture is the primary process affecting N₂O emissions associated with N fertiliser applications to soils.

The inclusion of UI did not influence either the average daily N₂O flux or the cumulative emission from surface applied urea in our study, similar to the findings of Carswell et al. (2019), Wallace et al. (2018). However, other studies have reported significantly lower cumulative N₂O emissions from urea treated with NBPT, with mean reduction ranging between 35% to 86% (Abalos et al., 2012; Ribeiro et al., 2020; Sanz-Cobena et al., 2012; Tian et al., 2015). In Sanz-Cobena et al. (2016), the decrease in N₂O emission was linked to the reduction in soil NH₄⁺ concentration as a result of NBPT inclusion, suggesting that nitrification was the main process of N₂O production in these soils. A positive correlation between soil NH₄⁺ concentration and N₂O emission has been reported in studies where UI effectively mitigated N₂O emissions (Abalos et al., 2012; Sanz-Cobena et al., 2016). By reducing the NH₄⁺ concentration, NBPT limits the NO₃⁻ supply for denitrification, and may also decrease the soil denitrification potential as observed by Abalos et al., (2012). In our study, soil NH₄⁺ concentration was measured

only at the end of incubation, but the lack of a significant difference between the NH_4 concentration under urea alone and urea treated with UI (US and UL) could explain why UI did not influence N_2O emission. Although, UI effect on N_2O emissions following fertilisation with urea was not observed in this study, indirect N_2O emissions from NH_3 emissions would be reduced by inclusion of UI, due to the reductions in NH_3 emissions as reported elsewhere (Martins et al., 2017; Hu et al., 2020).

Losses of the applied urea-N were significantly greater as NH_3 than N_2O under all temperature and soil moisture conditions in our incubation study. In addition, our results suggest that both UI (US and UL) can significantly abate NH_3 emissions and may offer a potential mitigation option for NH_3 volatilisation from urea-fertilised soils under high temperatures, although the longevity and efficacy of the UI may be lower than under more temperate conditions. Suter et al. (2011) suggested that a higher concentration of NBPT may be required under conditions $>25^\circ\text{C}$ in irrigated pasture in Australia. NBPT concentrations up to 1000 ppm have been shown to enhance UI efficacy under tropical climatic conditions in Brazil (Mira et al., 2017), although other studies have shown no benefit in reducing emission or increasing crop yield of increasing the NBPT concentration above the commercially recommended concentration of c. 500 mg kg^{-1} (Antisari et al., 1996; Silva et al., 2017; Watson et al., 2008). However, these studies were conducted under more temperate conditions, and an assessment of higher NBPT concentration under relevant agroecosystems experiencing high temperature and low moisture conditions is warranted. Furthermore, as nitrification was likely the dominant pathway for N_2O emissions from our soil at WFPS below 60%, an assessment of nitrification inhibitors under these conditions may also be relevant in a warmer and drier climate.

2.7 Conclusions

Soil temperature and moisture influenced NH_3 emissions from all urea fertiliser treatments, with highest emission occurring at the highest soil temperature (35°C) and lowest soil moisture (20% WFPS). Inclusion of either UI (US and UL) with urea significantly and equally decreased average daily NH_3 flux and cumulative emission under all temperature and moisture conditions. However, the effectiveness of the UI to reduce NH_3 emissions declined as temperature increased and as soil moisture decreased.

Ammonia emission continued for longer following urea application at 35°C (up to 25 days) than at 15 or 25°C (up to 14 days). Nitrous oxide emission was not affected by either UI. However, the combined effect of soil moisture and temperature influenced N₂O-N emissions with higher cumulative emissions measured under low moisture (40% WFPS, favourable for nitrification) at 15 and 25°C, but higher at 60% WFPS at 35°C. The results suggest treatment of urea with UI is a promising mitigation strategy for reducing NH₃ emissions in warmer and drier climates, however the reduction in its efficacy may be an area for further refinement in a smart fertiliser.

2.8 Author contributions

Marieme Drame: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing-Original Draft, **Alison Carswell:** Conceptualization, Methodology, Visualization, Writing-Reviewing and Editing, Supervision, **William Roberts:** Investigation, **Jess Hood:** Formal analysis, **Martin Jemo:** Writing-Review & Editing, **Sigrid Heuer:** Writing-Review & Editing, **Mark Pawlett:** Writing-Review & Editing, **Guy Kirk:** Writing-Review & Editing, Supervision, **Tom Misselbrook:** Validation, Data curation, Writing-Reviewing and Editing, Supervision.

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3 Nitrogen fertiliser Forms Influence on Wheat Nitrous Oxide Emissions

3.1 Abstract

Nitrous oxide (N_2O) emissions' adverse environmental impacts are explored mainly in soil emitted sources, N-based fertiliser application forms and rates. During their growth development stage, the plant emits N_2O through the nitrate assimilation pathway. Plant species variety and photosynthetic activity influence the observed extent. However, little is known about the influence of the form of nitrogen (N) supplied to the plant and different fertiliser types. This study assessed the influence of fertiliser forms on plant N assimilation and N_2O emission in two spring wheat varieties (Cadenza and Sokoll). Plants were grown in soil and hydroponic solution at high or low N levels before adding the fertiliser. In the soil, fertiliser N was applied as urea, urea with N-(n-butyl) thiophosphoric triamide (NBPT), potassium nitrate (KNO_3), and a control with no additional N. In the hydroponic experiment, the fertilisers applied were KNO_3 , KNO_3 with a nitrate reductase (NR) inhibitor, KNO_3 with a glutamine synthetase (GS) inhibitor, KNO_3 with sodium nitroprusside (SNP) and a zero additional N control. In the soil experiment, N_2O emission from plant shoots was numerically more significant than from bare soil, although the differences were insignificant. Plant emission was more significant when KNO_3 was supplied, particularly under low N levels. Wheat variety Sokoll had lower GS activity and higher N_2O emission when KNO_3 was added compared to Cadenza. Plant emission was comparable to soil emission in hydroponics, though the difference from the solution alone without plant was insignificant. NR inhibitors did not affect leaf nitrate concentration and NR activity, whereas GS activity was reduced with a subsequent increase in ammonium concentration. The measured leaf N components were also higher at high N than low N levels. The study unequivocally refutes past claims of N_2O emissions from plants. Instead, it asserts that plant GS activity might directly or indirectly impact the N_2O emission.

Keywords: Nitrogen metabolism, Wheat variety, Fertiliser, Gas emission, Plant nutrient

3.2 Introduction

Agriculture contributes over 50% of nitrous oxide (N₂O) emissions from anthropogenic sources (Tian et al., 2020), with increased utilisation of nitrogen (N) fertilisers being the principal source of this (Smith, 2017). In cropping systems, the primary source of direct N₂O emissions is microbially driven nitrification and denitrification processes in soils (Hassan et al., 2022) and represent about 70% of N₂O emissions (Butterbach-Bahl et al., 2013; Lenhart et al., 2019). Plants can affect N₂O emissions by inducing changes in soil characteristics such as pH, carbon, oxygen and N availability, influencing nitrification and denitrification processes (Hayashi et al., 2015; Philippot et al., 2009). Some recent studies have suggested that plant aboveground-biomass is also an N₂O source (Hakata et al., 2003; Lenhart et al., 2019). Although others have reported plants to only act as a conduit to transport N₂O emitted belowground by soil microorganisms through root uptake of soil solution N₂O, which is transferred to the leaf via the transpiration channel and then released to the atmosphere (Bowatte et al., 2014; Ferch and Römheld, 2001). Machacova et al. (2016) reported that 18% of forest N₂O emissions originated from the *Pinus sylvestris* L. trees. Significant global N₂O emissions (0.20 – 0.37 Tg yr⁻¹) were also estimated from Cryptogamic covers (lichens and mosses) in various environments in Germany, Australia, Sweden and New Zealand (Lenhart et al., 2015).

Nitrous oxide emission mechanisms from plant leaves are not well known; some studies have reported that nitrate (NO₃⁻) is the precursor of plant-derived N₂O produced through NO₃⁻ reduction and assimilation in leaves during photosynthesis (Goshima et al., 1999; Hakata et al., 2003; Smart and Bloom, 2001). For instance, studies on aseptically grown plants and axenic microalgae cells supplied with NO₃⁻ supported N₂O production inside plant cells (Bauer et al., 2016; Hakata et al., 2003; Timilsina et al., 2020). Furthermore, laboratory studies using stable isotopes reported ¹⁵N₂O production from 34 plant species, including tobacco, maize, and citrus (Lenhart et al., 2019). However, Lenhart et al. (2019 and 2015) found that plant N₂O emission was not influenced by light intensity (day or night), suggesting a different plant pathway mechanism might produce N₂O from NO₃⁻ when photosynthesis is not occurring.

Plant N₂O emissions are influenced by increasing air temperature, which increases N₂O emissions from lichens, mosses, and tobacco plants (Lenhart et al., 2019, 2015).

Variations in plant N₂O emission have also been linked to genotype differences in phenological phases (flowering stage) in wheat (Karmakar et al., 2014). In comparison, Borah and Baruah (2016) attributed differences in wheat N₂O emission to physiological processes (i.e., transpiration) and anatomical features (i.e., stomatal frequency of adaxial flag leaf surface and xylem size). Genotype differences (e.g., root and shoot weight) in rice N₂O emission were also reported by Baruah et al. (2010) and Ma et al. (2012).

The use of urease inhibitors (UI) in agriculture aims to improve the efficiency of urea as an N fertiliser by reducing ammonia (NH₃) volatilisation and increasing the longevity of N in the soil, thereby increasing the time N is available to plants. Although the action of UI is aimed at soil urease activity, several authors have reported impediments in plant urease activity, in root uptake of urea and plant N assimilation in the presence of the UI N-(n-butyl) thiophosphoric triamide (NBPT; Artola et al., 2011; Cruchaga et al., 2011; Zanin et al., 2016, 2015). These impediments in plant N metabolism include the decline of glutamine synthetase (GS) activity (Artola et al., 2011; Cruchaga et al., 2011), which has been found to affect plant photosynthesis at different N rates (Fuentes et al., 2001) and has a potential influence on plant N₂O emission (Oszvald et al., 2022). However, there needs to be more investigation into whether NBPT influences plant-related N₂O emission and through what mechanism. An improved understanding of this under different fertiliser management regimes could highlight areas for enhancing crop N use efficiency.

This study investigated whether UI application with urea fertiliser affects wheat N assimilation and plant-based N₂O emission under two soil N levels. Furthermore, we applied GS activity, NO₃⁻ reductase (NR) inhibitors, and sodium nitroprusside (SNP) in hydroponic culture to identify the wheat N₂O production pathway.

3.3 Materials and Methods

3.3.1 Plant and soil material

Two experiments were conducted at Rothamsted Research, Harpenden, UK: one in a glasshouse with pot-grown plants in soil with the spring wheat varieties Cadenza, from the UK, and Sokoll, from the International Maize and Wheat Improvement Center

(CIMMYT). The second was conducted in a controlled environment cabinet, with the spring wheat variety Cadenza, grown in hydroponic solution.

3.3.2 Experiment in soil

Seeds were surface sterilised with 100% ethanol for 2 minutes and 5% bleach for 15 minutes (Oszvald et al., 2022). After washing five times with sterile water, the seeds were soaked in sterilised water overnight at 4°C in the dark. Seeds were germinated on wet filter paper at room temperature (RT) for 7 days. Single seedlings were transferred into 13 cm × 10 cm × 11 cm deep plastic pots containing Rothamsted Premix (75% peat, 12% screened sterilised loam, 3% vermiculite, 10% grit; Petersfield Products, Leicester, UK). The pots were fertilised with a modified Letcombe solution (Drew and Saker, 1984) 24 h after seedling transfer. Low and high initial soil N levels were established by applying 7 and 70 kg N ha⁻¹ of KNO₃ for plant growth. Growth conditions: 24°C/21°C(day/night), 16 h light at > 400 μmol m⁻² s⁻¹ (supplementary light by high-pressure sodium (SON-T) lighting as required) and 8 h darkness. Plants were watered daily from the surface to provide sufficient water for growth.

After five weeks, three additional N treatments were applied at an equivalent rate of 250 kg N ha⁻¹ to create conditions for detectable emissions: (1) as urea (hereafter U; Diamond Fertilisers, Lincolnshire, UK); (2) as urea with the UI NBPT (US; Origin SUSTAIN®, Hertfordshire, UK) at 600 ppm; KNO₃; and (3) a control with no additional N (hereafter termed C). For a set of pots, the aboveground plant biomass were removed from 10 mm above the soil surface to assess emission from the soil and roots (roots and soil pots). A further set without plants and no additional N was also prepared to assess the soil-only emission (Soil pots). Empty chambers were used to assess ambient N₂O concentration. All treatments were replicated six times. The experiment was laid out in a factorial design. Gas measurements began immediately after the fertiliser treatments (Section 3.3.4).

3.3.3 Experiment in hydroponics

Cadenza seedlings were germinated as described in Section 2.2. Single seedlings were transferred to 1 L aerated hydroponic culture pots (covered glass containers) containing modified Letcombe solution, adjusted to pH 5.8 with KOH. The plants were grown under

high and low N levels as the soil-grown plants. The basic nutrient solution composition per litre for the high N was 1.5 mM $\text{Ca}(\text{NO}_3)_2$, 5 mM KNO_3 , 2 mM NaNO_3 , 1.5 mM MgSO_4 , 1 mM phosphate buffer solution ($\text{KH}_2\text{PO}_4 - \text{K}_2\text{HPO}_4$, pH 6.0), 50 μM FeNaEDTA , 0.5 μM CuCl_2 , 20 μM H_3BO_3 , 3.6 μM MnCl_2 , 0.1 μM Na_2MoO_4 and 0.77 μM ZnCl_2 . Additionally, 0.25-0.5 g/L MES monohydrate (Alfa Aesar, Massachusetts, USA) was added as a pH buffer. The low N level had 10 times less N, and N was supplied as 0.1 mM KNO_3 to maintain the same ionic strength as in the high N solution, $\text{Ca}(\text{NO}_3)_2\text{H}_2\text{O}$ replaced by CaCl_2 , KNO_3 by KCl and NaNO_3 by NaCl . The nutrient solution was changed three times a week. Growth conditions: 24°C/21°C (day/night) with 16 h daylight at 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 8 h darkness in a Gallenkamp 228 (Sanyo, Osaka, Japan) controlled environment cabinet.

After 5 weeks, supplemental N was applied at 40 mM L^{-1} as (1) KNO_3 only, (2) KNO_3 and sodium nitroprusside ($\text{KNO}_3\text{-SNP}$), (3) KNO_3 and NR inhibitor ($\text{KNO}_3\text{-NR}$), (4) KNO_3 and methionine sulfoximine as GS activity inhibitor ($\text{KNO}_3\text{-MSO}$) and (5) control with zero additional N (C). The SNP, NR and MSO were applied at 100 $\mu\text{M L}^{-1}$. Unplanted pots with only deionised (DI) water were used as a solution control. The experiment was laid out in a complete randomized block design with five replicates of each treatment. Gas measurements began immediately after the N treatments (Section 3.3.4).

3.3.4 Gas measurements

For the experiment in soil, immediately after N fertiliser treatments, in the planted pots (with shoots) and roots+soil pots (stems removed) and unplanted soil pots, the soil surface was covered with aluminium foil overlapping the pot edges and covering the base of the plant stems, and then cling film was wrapped around the plant stems and overlapping the pot edges to provide a near gas-tight seal. This approach was adapted from that applied using the static chamber method to maximise the opportunity to detect a flux from the plants. A 10-cm diameter, 15-cm tall plastic extension chamber was placed on top of the pot and sealed with adhesive PVC insulation tape. A clear acrylic gas sampling chamber was fitted to the extension chamber, and the chamber inlet and outlet tubes were connected to a gas analyser via a pump. The total headspace volume was 3,393 cm^3 .

For the hydroponic experiment, the insertion point of the air tubing on the top of the culture pots was sealed with adhesive tape prior to fixing the extension chamber and Picarro gas sampling chamber on top, as for the soil-grown plants. The total headspace volume was 3,110 cm³.

For both the experiments in soil and hydroponics, the air in the chamber headspace was circulated through a Picarro G2508 gas analyser (Picarro Inc., Santa Clara, CA, United States) at 250 mL min⁻¹ using a low-leak external pump, and N₂O concentration was measured. The Picarro instrument was connected to a multiplexer (Picarro 16-Port Distribution Manifold A0311) set up to measure 16 chambers for 3 minutes 49 seconds each, consecutively and repeatedly over 24 hours. Concentrations of N₂O (dry molar mixing ratio) were recorded on average every 2 s. Gas measurement over the 24 hours was done to optimise the detection of flux from the plants, although, the limitations for leaving the chamber lids in place for 24 hours for measuring soil fluxes are recognised.

N₂O emission was measured over 10 and 5 days for the soil and hydroponic experiments to allow measurement of all replicates, respectively. The N₂O emission for each monitored pot was calculated over the first 6–10 h of measurement after excluding the first 15 s for each sampling time, assuming a linear increase in the measured chamber concentration:

$$E = V \frac{dC}{dt} \quad (3-1)$$

where E is N₂O emission (ug N₂O-N plant h⁻¹), V is the internal chamber volume (m³), C is the N₂O concentration in the chamber (ppm), and t is the time of sampling. dC/dt is the rate of change in C over time obtained from the slope of a linear regression of C against t .

3.3.5 Plant sampling and analysis

After measuring N₂O for 24 h, measurements were stopped, and the chambers were removed. The most developed plant leaves were destructively sampled, frozen in liquid N and stored at -80°C before analysis. The remaining above-ground biomass was harvested, dried at 80°C for 24 h and weighed. The frozen leaf tissues were finely ground in liquid N, and tissue NH₄⁺, and protein concentration and GS activity were determined

with a microplate spectrophotometer (SPECTRAmax® 340PC; Molecular Devices Corporation, California, USA). A 100 mg fresh leaf sample was mixed with 1 ml of 0.05 M sulfuric acid, incubated at RT for 1 h (Mattsson et al., 1997), and then centrifuged at 13,000 RCF for 15 min. The supernatant was sampled to measure leaf NH_4^+ concentrations using the Megazyme rapid ammonia kit (K-AMIA 02/21). Protein concentration was determined following the method of Bradford, (1976), involving the binding of Coomassie Brilliant Blue G-250 to protein. A 100 mg sample of fresh leaf tissue was mixed with 1 ml of buffer solution (containing 25 mM TRIS-HCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Dithiothreitol, 1 mM Magnesium Chloride, 0.2 g polyvinyl pyrrolidone and 2 mM leupeptin, pH 7.6 (Oszvald et al., 2022), GS activity was then measured according to O'Neal and Joy, (1973) by incubating 50 μl leaf extracts in an assay medium containing 600 mM glutamate, 45 mM hydroxylamine hydrochloride, 150 mM magnesium sulphate heptahydrate, 30 mM EDTA disodium salt, and 60 mM adenosine triphosphate for 30 min at 30°C in a shaker incubator. The enzyme reaction was terminated with 300 μl stopping solution (0.67M FeCl_3 , 0.2M TCA, 0.7M HCl).

The leaf NO_3^- concentration was measured in water extracts of 50 mg ground leaf tissue following Zhao and Wang, (2017). Leaf tissue samples were mixed with 100 μl of deionised (DI) water and heated at 100°C for a minimum of 20 min. The extract was centrifugation at $15,871 \times g$ for 10 min. The supernatant was then put into a new tube, and 40 μl of 5% (w/v) salicylic acid-sulphuric acid was added. The tubes were incubated at RT for 20 min prior 0.95 ml of 8% (w/v) NaOH solution was added, and tubes were left to cool to RT for 20-30 min. The OD_{410} (absorbance) values were measured in a spectrophotometer, and DI water was used as a blank and as control. The NO_3^- content, Y ($\mu\text{g g}^{-1}$) was calculated as:

$$Y = \frac{CV}{W} \quad (3-2)$$

C is NO_3^- concentration calculated with OD_{410} using a regression equation from the standard curve ($\mu\text{g ml}^{-1}$), V is the total volume of the extracted sample (ml), and W is the weight of the sample (g).

Nitrate reductase activity was assayed in leaves using the method described by Kim and Seo, (2018). Extraction buffer containing 250 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 μ M Na₂MoO₄, 5 μ M flavin adenine dinucleotide (FAD), 3 mM dithiothreitol were added to 100 mg of frozen leaf powder and centrifuged at 12,000 g for 15 min at 4°C. Aliquots (50 μ l) of supernatants were added to 100 μ l reaction medium (5 mM KNO₃, 0.2 mM NADH) and incubated for 15 min at 25°C. Added zinc acetate (100 μ l, 0.5 M) stopped the reaction. The mixture was centrifuged at 8000 RCF for 5 min and the supernatant was used to determine NO₃⁻ production after the addition 150 μ l of Greiss reagent (2% sulphanylamide in 5% H₂PO₄ and 0.1% 1-Naphthyl ethylenediamine dichloride) and reading the solution absorbance at 540 nm (Lambda 25 UV/VIS Spectrometer, Waltham, USA). The amount of NR required to produce 1 μ mol NO₃⁻ per minute was defined as one unit of the NR. Plant total N (%) was measured from the dried biomass using a LECO TruMac combustion analyser (Stockport, UK).

3.3.6 Statistical analysis

The nutrient level, fertiliser type, and effects on N₂O emission, tissue NH₄⁺, NO₃⁻, soluble protein concentration, GS and NR activity, and plant dry weight were evaluated using a linear mixed model regression. The treatment structure had N₂O source, wheat variety, fertiliser type and soil N level as fixed effects and the sampling date as a random effect. N₂O sources include ambient air, pots with shoots, roots+soil, and soil only. The hydroponic experiment N₂O analysis treatment structure had N₂O source, fertiliser type and solution N level as fixed effects and sample position (row position) in the cabinet as a random effect. Nitrous oxide sources include ambient air, pot with shoots, and DI water only. For the analysis of leaf N components, the factor N₂O source was removed from the fixed effect structure for both experiments. Pairwise data comparisons were made using the least significant difference, with differences between treatments considered significant where $p < 0.05$. To meet all model assumptions of data normality, the N₂O data was transformed into square roots. Two outliers were identified from the soil experiment N₂O data with variance shift outlier model in GenStat (22nd edition) and removed before statistical analysis. Leaf NH₄⁺ concentration and GS activity in the hydroponic experiment were transformed by log and square root, respectively. Leaf GS

activity in the soil experiment was log-transformed. Statistical analyses were carried out using GenStat (22nd edition) statistical software.

3.4 Results

3.4.1 Soil experiment

3.4.1.1 Nitrous oxide emission

Nitrous oxide emissions measured from the four sources (soil, ambient air, roots+soil and shoots) were not significantly different from each other, although shoots had, numerically, the greatest emission (Figure 3-1a). Application of KNO₃ significantly increased N₂O-N emission (Table 3-1), particularly under low N levels (Figure 3-1b). The interaction effect of source, variety and fertiliser type was significant, with significantly greater N₂O-N emission in the shoots of the Sokoll plant treated with KNO₃ than in Cadenza (Figure 3-1c). There was no significant effect of fertiliser type for pots with roots and soil (Supplementary information Figure C-1).

Table 3-1: Effect of emission source, fertiliser type, and nutrient level on nitrous oxide emission in soil experiment.

		N ₂ O (µg N ₂ O-N plant ⁻¹ h ⁻¹)
<i>Emission source</i>		
	Shoots	0.221±0.013 ^a
	Roots + Soil	0.212±0.014 ^a
	Soil	0.191±0.023 ^a
	Ambient	0.190±0.023 ^a
<i>Fertiliser type</i>		
	C	0.194±0.015 ^a
	U	0.205±0.015 ^a
	US	0.214±0.015 ^a
	KNO ₃	0.259±0.015 ^b
<i>Nutrient Level</i>		
	Low N	0.222±0.015 ^a
	High N	0.214±0.015 ^a
<i>Significance of fixed effects</i>		
Emission source		0.472
Fertiliser type		<0.001

Nutrient level	0.499
<i>Interaction effects</i>	
Emission source x Variety	0.319
Emission source x Fertiliser type	0.137
Emission source x Nutrient level	0.450
Fertiliser type x Nutrient level	0.038
Emission source x Variety x Fertiliser type	0.010
Emission source x Variety x Nutrient level	0.866
Emission source x Fertiliser type x Nutrient level	0.215
<u>Emission source x Variety x Fertiliser type x Nutrient level</u>	<u>0.069</u>
Values are predicted mean values $\pm$ standard error ($n = 6$) from linear mixed effects model analysis. Letters depict a significant difference between means within columns ($p < 0.05$).	

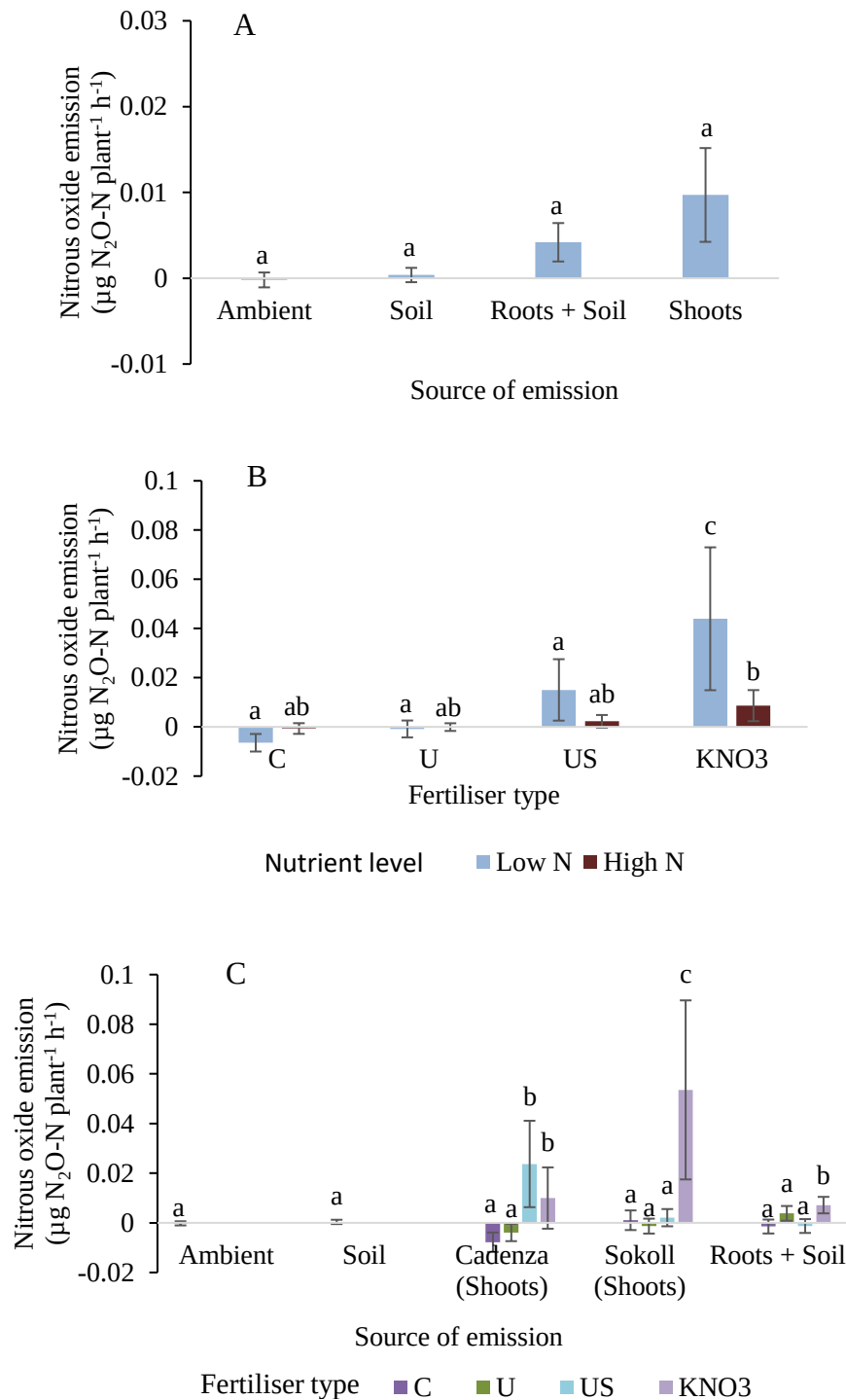


Figure 3-1: Nitrous oxide emission in the experiment in soil (a) from different sources; (b) from shoots as influenced by fertiliser type and N level; and (c) from shoots as influenced by wheat variety, averaged across fertiliser type. Bars are means $\pm$ standard error ($n = 6$). Letters depict significant differences between means ($p < 0.05$). The control treatment in (b) refers to emission from pots with no additional N (averaged from Shoots and Roots + soil) and (c) relates to Shoots emission with no additional N.

3.4.1.2 Leaf N components

Leaf NH_4 concentration and GS activity were influenced by variety (Table 3-2). The variety Sokoll had greater NH_4^+ concentration, whereas Cadenza had higher GS activity (Figure 3-2a and b, respectively). Additionally, GS activity varied in response to the interaction effect of variety and fertiliser type. Sokoll leaf GS activity was lower under U than US-treated plants (Figure 3-2c). Any applied treatment factors (variety, nutrient level, and fertiliser type) did not significantly influence leaf protein concentration. The interaction of wheat variety and N level significantly affected dry weight, with Sokoll's variety observing greater dry weight than the Cadenza under high N growth conditions. In contrast, Cadenza's dry weight was significantly greater than Sokoll's under low N growth conditions (Figure 3-3). Additionally, in Sokoll, dry weight was higher under high compared to low N growth levels, whereas for Cadenza, there was no significant difference between high and low N growth levels.

Table 3-2: Leaf nitrogen components in response to Effect of variety, fertiliser type and nutrient level on wheat leaf nitrogen components, for plants grown in soil experiment.

		NH_4^+ ($\mu\text{mol g}^{-1}$)	GS ($\mu\text{g GHA mg FW}^{-1} \text{h}^{-1}$)	Protein (mg g^{-1})	Dry weight (g)
<i>Variety</i>					
	Cadenza	3.39±0.655 ^a	4.29±0.071 ^b	0.491±0.023 ^a	0.312±0.055 ^a
	Sokoll	5.24±0.619 ^b	3.97±0.063 ^a	0.476±0.020 ^a	0.312±0.055 ^a
<i>Fertiliser type</i>					
	C	3.88±0.876 ^a	4.11±0.110 ^a	0.506±0.035 ^a	0.301±0.059 ^a
	U	4.98±0.701 ^a	4.00±0.079 ^a	0.449±0.024 ^a	0.352±0.056 ^a
	US	4.41±0.682 ^a	4.16±0.076 ^a	0.462±0.023 ^a	0.315±0.056 ^a
	KNO ₃	3.99±0.962 ^a	4.25±0.118 ^a	0.517±0.034 ^a	0.280±0.061 ^a
<i>Nutrient Level</i>					
	High N	4.07±0.762 ^a	4.23±0.062 ^a	0.468±0.020 ^a	0.318±0.076 ^a
	Low N	4.56±0.762 ^a	4.03±0.060 ^a	0.499±0.021 ^a	0.306±0.076 ^a
<i>Significance of fixed effects</i>					
	Variety	0.009	0.011	0.473	0.999
	Fertiliser type	0.715	0.428	0.171	0.110
	Nutrient Level	0.583	0.144	0.585	0.963
<i>Interaction effects</i>					
	Variety x Fertiliser type	0.383	0.042	0.246	0.922
	Variety x Nutrient level	0.739	0.328	0.465	<0.001
	Fertiliser type x Nutrient level	0.936	0.493	0.268	0.717
	Variety x Fertiliser type x Nutrient level	0.662	0.398	0.752	0.642

Values are predicted mean values ± standard error ($n = 6$) from linear mixed effects model analysis. Letters depict a significant difference between means within columns ($p < 0.05$). GHA is γ -glutamyl hydroxamate and FW is fresh weight.

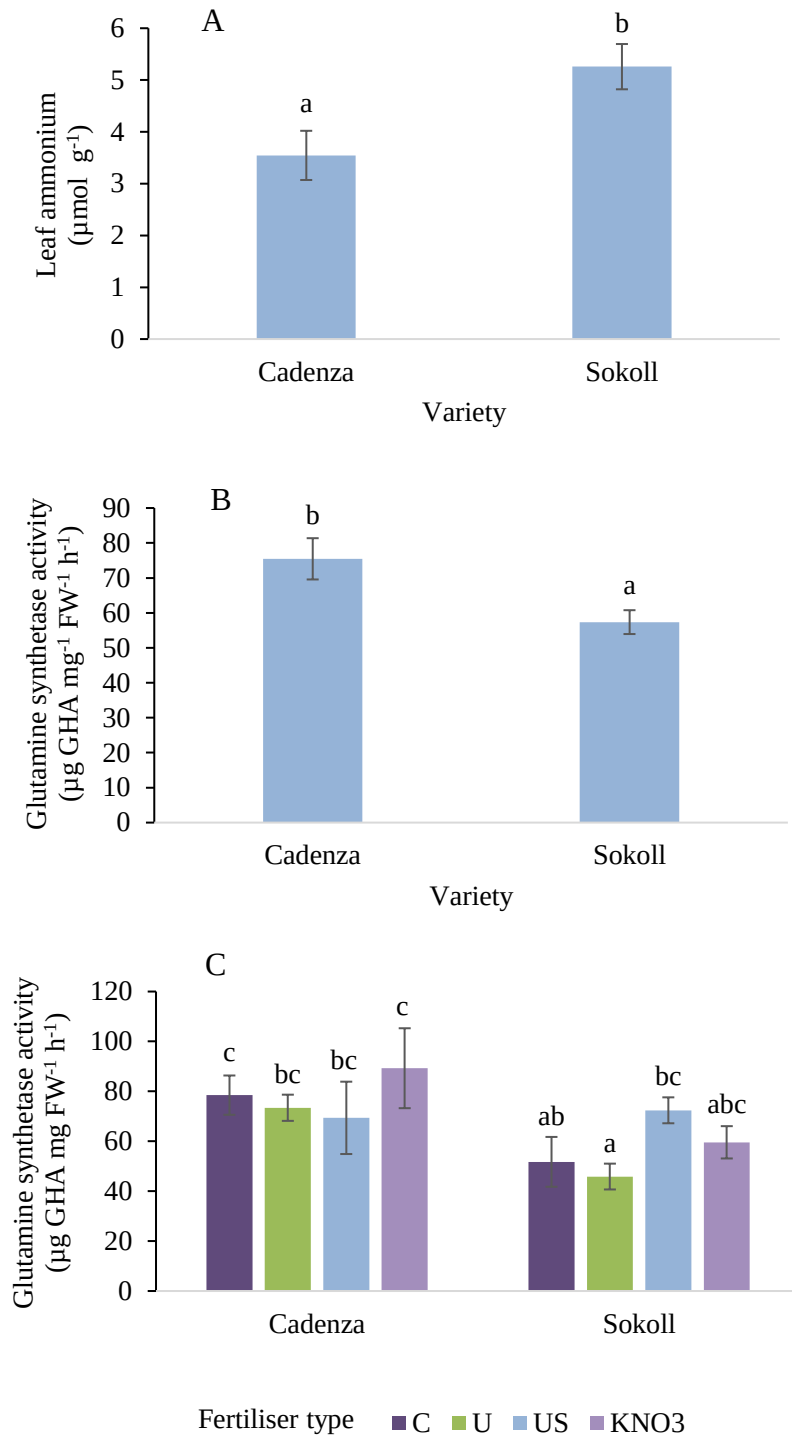


Figure 3-2: Leaf NH_4^+ concentrations in the experiment in soil as influenced by (a) wheat variety. Leaf GS activity as influenced by (b) variety; and (c) variety averaged across fertiliser type. Bars are means $\pm$ standard error ($n = 6$). Letters depict significant difference between means ($p < 0.05$). GHA is γ -glutamyl hydroxamate and FW is fresh weight. The control treatment here refers to shoots with no additional N.

3.4.2 Hydroponic experiment

3.4.2.1 Nitrous oxide emission

The N₂O emission from plants grown in hydroponics was comparable to that from the plants grown in soil, of the order of 0.1 µg N₂O-N plant⁻¹ h⁻¹ (Figure 3-4a). However, owing to the large variance in the plant emission data, the emission was not significantly different from that of the ambient air or solution (Figure 3-4a; Table 3-3). There was no significant effect of the applied N level or the fertiliser type (Figure 3-4b) or their interactions (Table 3-2). However, plants that received KNO₃ numerically produced more N₂O than the other N forms.

Table 3-3: Effect of emission source, fertiliser type, and nutrient level on nitrous oxide emissions from wheat plants grown in hydroponic experiment.

		N ₂ O (µg N ₂ O-N plant ⁻¹ h ⁻¹)
<i>Emission source</i>		
	Ambient	0.117±0.027 ^a
	No plant	0.112±0.027 ^a
	Plant	0.133±0.009 ^a
<i>Fertiliser type</i>		
	C	0.134±0.019 ^a
	KNO ₃	0.159±0.019 ^a
	KNO ₃ - MSO	0.124±0.019 ^a
	KNO ₃ - NR	0.132±0.019 ^a
	KNO ₃ - SNP	0.114±0.019 ^a
<i>Nutrient Level</i>		
	High N	0.135±0.012 ^a
	Low N	0.130±0.012 ^a
<i>Significance of fixed effects</i>		
Emission source		0.677
<i>Interaction effects</i>		
Emission source x Fertiliser type		0.530
Emission source x Nutrient level		0.747
Emission source x Fertiliser type x Nutrient Level		0.729

Values are predicted mean values ± standard error ($n = 5$) from linear mixed effects model analysis. Letters depict a significant difference between means within columns ($p < 0.05$).

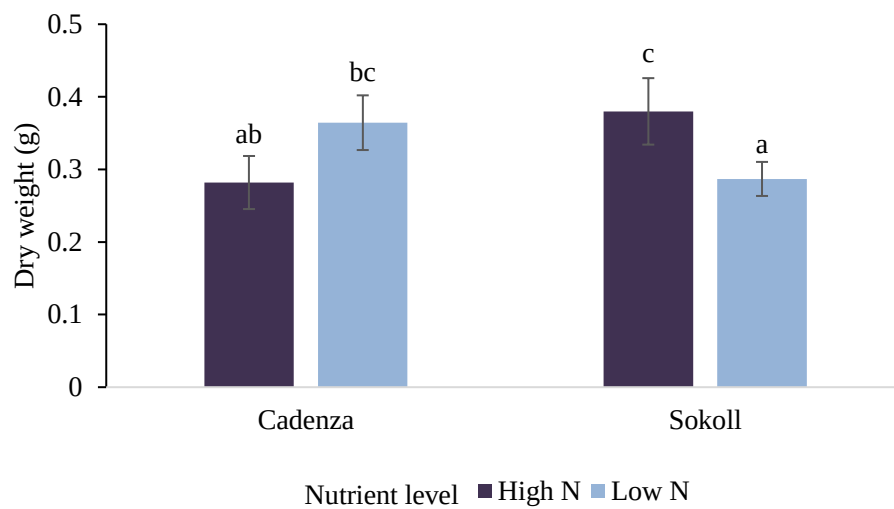


Figure 3-3: Dry biomass weigh of one wheat plant, as influenced by variety and averaged across nitrogen level in the experiment in soil. Bars are means $\pm$ standard error ($n = 6$). Letters depict significant difference between means ($p < 0.05$).

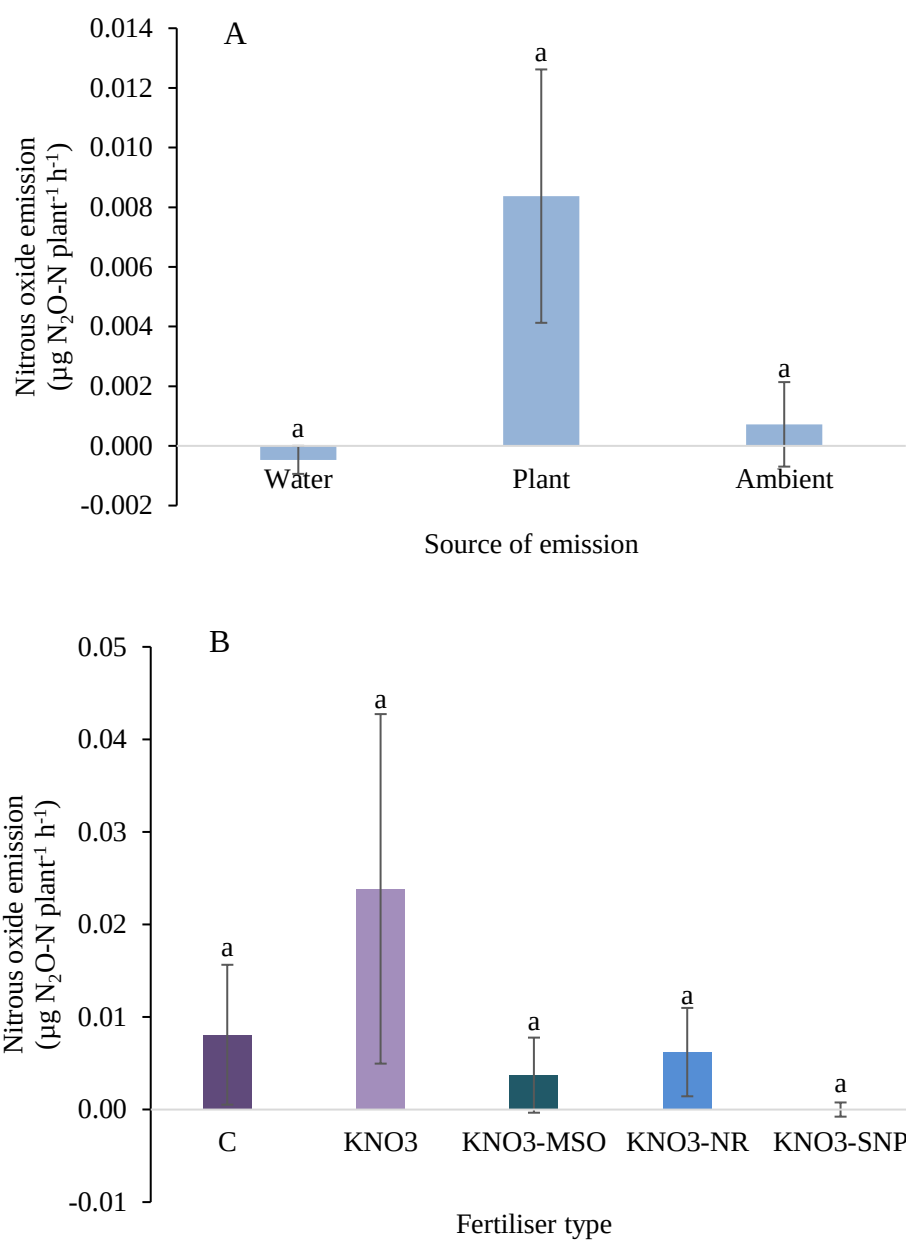


Figure 3-4: Leaf $\text{N}_2\text{O-N}$ emission in the hydroponic experiment (a) from different emission sources; and (b) as influenced by fertiliser type. Bars are means $\pm$ standard error ($n = 5$). Letters depict significant difference between means ($p < 0.05$).

3.4.2.2 Leaf N components

The effect of N level during plant development significantly influenced all leaf N components, enzyme activity and dry weight, whereas fertiliser type only influenced NH_4^+ concentration and GS activity (Figure 3-5; Table 3-4). The combined effect of fertiliser type and N level was only observed for leaf NH_4^+ concentration (Table 3-4). Leaf NH_4^+ concentration was significantly greater in plants that received KNO_3 -MSO and greater at low N levels (Figure 3-5a). Whereas leaf GS activity was significantly smaller in plants treated with KNO_3 -MSO and greater at high N levels (Figures 3-5b and 3-5c, respectively). Leaf NO_3^- concentration and NR activity were greater under high N (Figures 3-6a and 3-6b). Leaf total N, protein concentration, and plant dry weight were greater at high N levels (Figures 3-6 c, 3-6d and 3-6e). Supplying KNO_3 -NR or KNO_3 -NSP did not influence leaf N components.

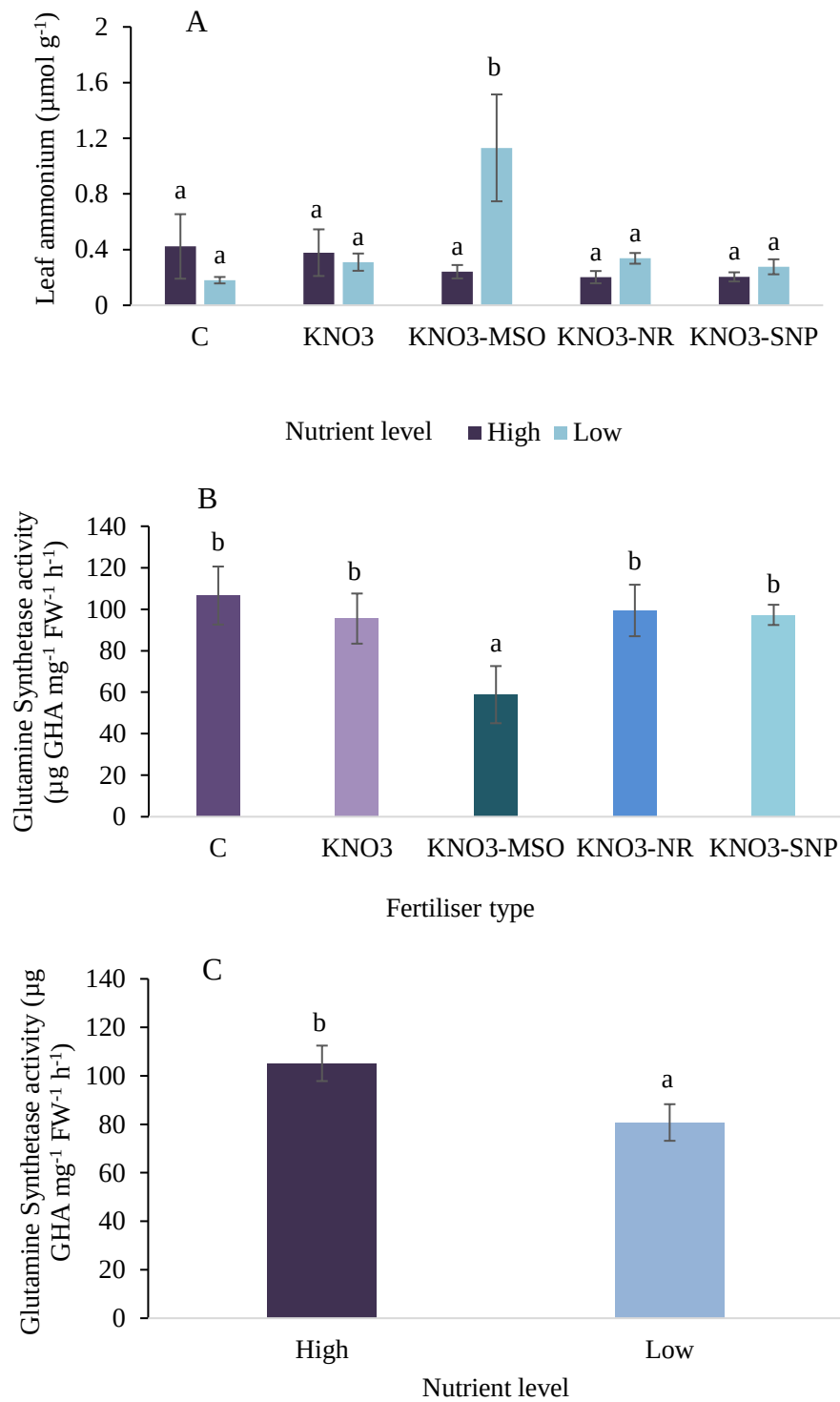


Figure 3-5 : Leaf NH_4^+ concentrations in the hydroponic experiment (a) as influenced by fertiliser type averaged across nutrient level. Leaf glutamine synthetase activity as influenced by (b) fertiliser type; and (C) by nutrient level. Bars are means $\pm$ standard error ($n = 5$). Letters depict significant difference between means ($p < 0.05$). GHA is γ -glutamyl hydroxamate and FW is fresh weight.

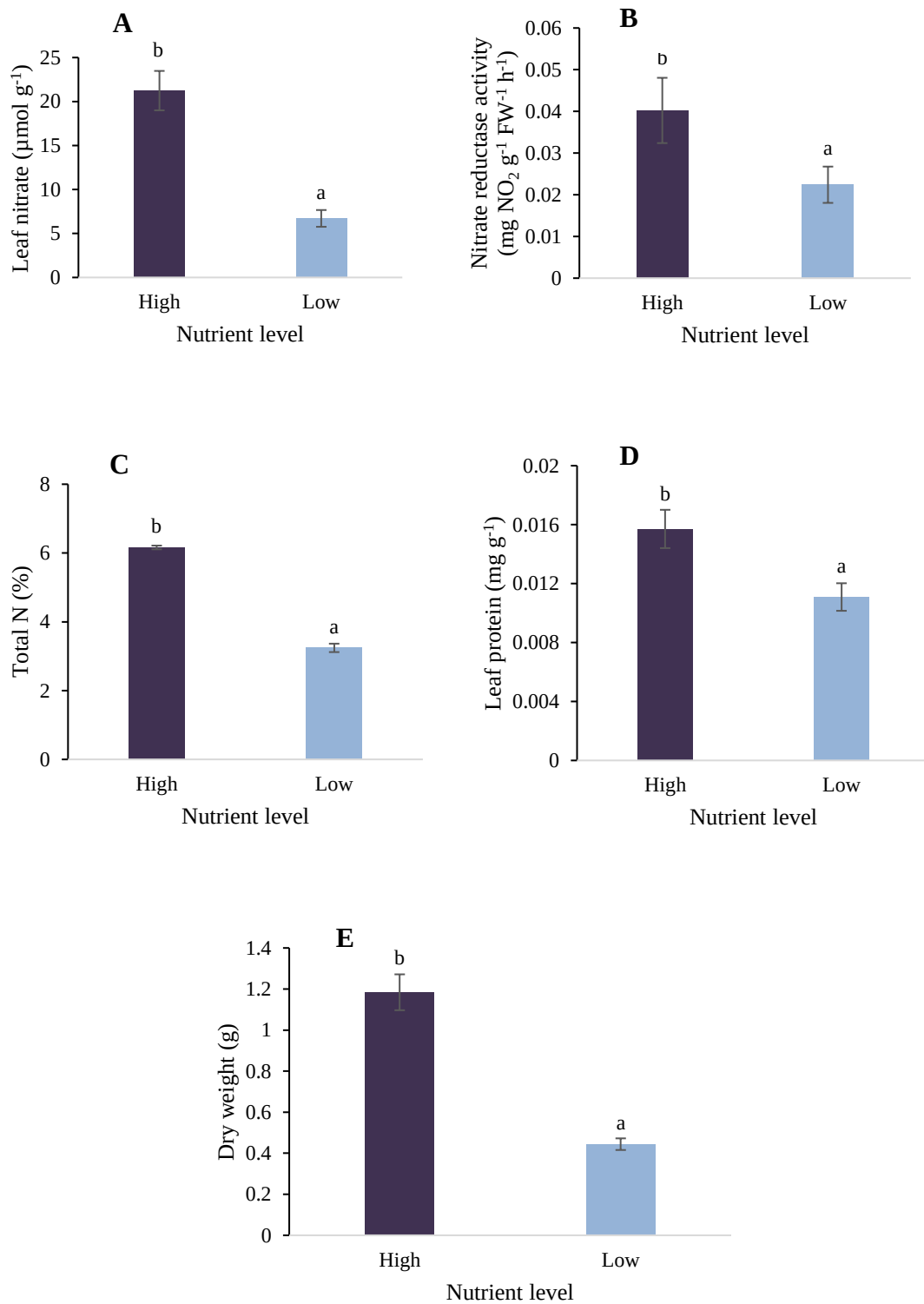


Figure 3-6: Influence of nitrogen level during wheat growth in the hydroponic experiment on (a) leaf NO_3^- concentrations; (b) leaf nitrate reductase activity; (c) leaf total N; (d) leaf protein; and (e) plant dry weight. Bars are means $\pm$ standard error ($n = 5$). Letters depict significant difference between means ($p < 0.05$). FW is fresh weight.

Table 3-4: Wheat leaf N-components according to linear mixed model effects in response to fertiliser type and nutrient level in hydroponic experiment.

	NH ₄ ⁺ (μmol g ⁻¹)	GS (μg GHA mg ⁻¹ FW ⁻¹ h ⁻¹)	NO ₃ ⁻ (μmol g ⁻¹)	NR (mg NO ₂ g ⁻¹ FW ⁻¹ h ⁻¹)	Total N (%)	Protein (mg g ⁻¹)	Dry weight (g)
Fertiliser type							
C	-1.43±0.247 ^a	10.1±0.514 ^b	10.7±2.87 ^a	0.030± 0.012 ^a	4.67 ± 0.168 ^a	0.016±0.002 ^a	0.640±0.096 ^a
KNO ₃	-1.26±0.219 ^a	9.76±0.547 ^b	11.5±2.73 ^a	0.029 ± 0.012 ^a	4.85 ± 0.168 ^a	0.012±0.002 ^a	0.887±0.096 ^a
KNO ₃ + MSO	-0.565±0.230 ^b	7.09±0.623 ^a	19.0±2.73 ^a	0.04 0± 0.012 ^a	4.49 ± 0.168 ^a	0.012±0.002 ^a	0.801±0.096 ^a
KNO ₃ + NR	-1.41±0.219 ^a	9.75±0.596 ^b	16.3±2.73 ^a	0.027 ± 0.012 ^a	4.78 ± 0.168 ^a	0.013±0.002 ^a	0.850±0.096 ^a
KNO ₃ + SNP	-1.51±0.230 ^a	9.88±0.514 ^b	12.0±2.87 ^a	0.032 ± 0.012 ^a	4.71 ± 0.168 ^a	0.015±0.002 ^a	0.884±0.096 ^a
Nutrient level							
Low N	-1.46±0.158 ^b	10.1±0.356 ^b	21.0±2.00 ^b	0.021 ±0.010 ^b	6.17 ± 0.127 ^b	0.011±0.001 ^b	0.438±0.055 ^b
High N	-1.02±0.169 ^a	8.56±0.329 ^a	6.81±1.94 ^a	0.042 ± 0.010 ^a	3.23 ± 0.127 ^a	0.016±0.001 ^a	1.19±0.055 ^a
<i>Significance of fixed effects</i>							
Fertiliser type	0.006	0.015	0.080	0.785	0.400	0.469	0.386
Nutrient level	0.017	0.008	<0.001	0.010	<0.001	0.003	<0.001
<i>Interaction of fixed effects</i>							
Fertiliser type x Nutrient level	0.004	0.489	0.671	0.225	0.408	0.291	0.188

Values are predicted mean values ± standard error ($n = 5$) from linear mixed effects model analysis. Letters depict significant difference between means within columns ($p < 0.05$). GHA is γ -glutamyl hydroxamate and FW is fresh weight.

3.5 Discussion

The experimental results did not provide evidence of direct N₂O emission from wheat leaf stomata. Nitrous oxide emissions measured in the current study were low with no significant increase in chamber headspace N₂O concentration from most of the measured pots, which may have reduced the probability of detecting significant differences. Low plant N₂O emission values were reported by Smart and Bloom (2001) with maximum leaf area emission $\leq 25.6 \text{ pmol m}^{-2} \text{ s}^{-1}$ from three leaf plants. N₂O emitted from N-treated shoots were numerically greater (averaged over wheat varieties and N fertiliser types) than from N-treated roots and soil, although not significantly different. This contrasts with observations by Smart and Bloom, (2001), where N₂O emission from wheat grown in hydroponic solution was measured simultaneously from both rhizosphere and shoots in separate sealed chambers and significantly higher N₂O emission was recorded in wheat shoots compared to roots and soil. Furthermore, a field experiment by Zou et al. (2005) gave insight into plant influence on N₂O emission in cropping systems using the cutting method (method description in Chen et al., 1999; Yan, 2000). Zou et al. (2005) estimated winter wheat biomass contribution to seasonal N₂O fluxes (fluxes ranged between 0.3 and 3.9 mg N₂O-N m⁻² day⁻¹) to an average of 25%, with the level of contribution dependent on wheat growth stage and ranging from 10 to 62% between tillering to heading stage, respectively.

Nevertheless, in the current study, the combined effect of wheat variety and fertiliser type on shoot N₂O emission suggested that emission was influenced by wheat variety and was probably related to wheat N uptake and shoot N assimilation. Supplying KNO₃ increased shoot N₂O emission in comparison to pots with roots + soil only, particularly under low N growth levels. Hakata et al. (2003), in a study on 11 plant species including wheat, revealed the ability of plants to convert ¹⁵KNO₃ into ¹⁵N₂O. The effect of KNO₃ in increasing shoot N₂O emission in our experiment suggests that the N₂O could derive from the NO₃⁻ reduction process. As observed in several studies, N₂O emission from plants is greater when the N fertiliser source is NO₃⁻, as it is the precursor of N₂O (Lenhart et al., 2019; Smart and Bloom, 2001). The measured N₂O emission in Smart and Bloom (2001) was derived from ¹⁵NO₃⁻ assimilation, particularly during NO₂⁻ reduction to NH₃ in the wheat chloroplast, which gave the observed strong relationship between the rate of

N₂O emission and NO₃⁻ assimilation quotient. This could explain the negligible N₂O emission from plants supplied with U and US fertilisers, which supply NH₄⁺ as the initial substrate, as reported elsewhere (Goshima et al., 1999; Lenhart et al., 2019). The hydroponic experiment in the present study did not give further information on the source of N₂O emission as the findings of Bowatte et al. (2014) in ryegrass fertilised with NH₄⁺ or NO₃⁻, which was attributed to the negligible contribution of the plant N₂O emission in fields. Additionally, using an NR inhibitor did not influence leaf NO₃⁻ concentration or NR activity in the 24 hours after application, with no noticeable effect on N₂O emission in contrast to Goshima et al. (1999) where N₂O emission from tobacco plants was restricted by NR inhibitor. Furthermore, supplying plants with additional NO through KNO₃-SNP did not influence N₂O emission, probably because NO reduction to N₂O in plants (mitochondria) is mainly catalysed under oxygen-limited conditions as described in Timilsina et al. (2020).

The greater N₂O emission observed from plants supplied with KNO₃ under low N growth levels was also reported by Oszvald et al. (2022). Schjoerring and Mattsson, (2001) also reported greater NH₃ emission from wheat under reduced N supply (75% of the recommended N dose), which they related to a greater dry biomass N concentration at the generative stage. Furthermore, Fuentes et al. (2001) reported that under N starvation conditions, transgenic tobacco lines tended to have higher photosynthesis and leaf area by over-expressing GS1 activity in cytosol compared to plants grown under optimum N conditions. Kumagai et al. (2011) reported increased rice photorespiration and NH₃ emission with increased GS activity. According to Zou et al. (2005), plant N₂O emission could be related to photosynthesis and respiration during dark hours. Observations by Schützenmeister et al. (2020) support the hypothesis that photosynthesis N-assimilation contributes to plant-derived N₂O emission in trees. Therefore, the influence of GS activity on plant photosynthesis productivity could influence plant gas emissions, including N₂O. Although, in the current study, particularly in the hydroponic experiment, all measured leaf N components (NH₄⁺, NO₃⁻ and protein concentration), enzyme activity (GS and NR), total N and plant dry weight were reduced under low N compared with high N growth levels, warranting further investigation into plant N₂O emissions (Oszvald et al., 2022)

The influence of KNO_3 on N_2O emission from the plant was more pronounced in Sokoll than in Cadenza. Sokoll had a lower GS activity, which is associated with the plant assimilation rate of NH_4^+ and the capacity of a plant to utilise N. After being taken up by the plant, NO_3^- is reduced to NH_4^+ and subsequently assimilated through GS activity into amino acids and protein (Balotf et al., 2016). Cadenza's greater N assimilation potential resulted in lower leaf NH_4^+ concentration and no significant change in dry biomass between high and low N growth levels compared to Sokoll. Oszvald et al. (2022) also reported higher N_2O emission from low GS activity varieties, although the relationship between GS activity and N_2O emission remains unclear. Inhibition of plant leaf GS activity in the hydroponic experiment did not clarify further as there was no effect on N_2O emission despite greater NH_4^+ concentration in the leaves. Further studies on the relationship between wheat GS activity and N_2O emission are needed. Bowatte et al. (2014) found significant differences between grass species in N_2O emission; however, their study did not support the hypothesis of direct plant N_2O emission from NO_3^- assimilation but suggested the plant acted as a medium to transport soil-derived N_2O , with plant species influencing soil microbial processes. Baruah et al. (2012) reported a positive correlation of N_2O emission with wheat transpiration rate in a field study, suggesting that wheat was a conduit for soil microbial N_2O . It is important to highlight that most studies reporting that plant N_2O emission was derived from the transport of soil N_2O did not simultaneously investigate plant N assimilation mechanisms (Baruah et al., 2012; Bowatte et al., 2014; Zou et al., 2005). Therefore, more studies on N_2O emission from plants are needed to bring more insight into the underlying mechanisms connecting plant GS activity, photosynthesis, respiration, and plant N_2O emission processes under different N growth environments.

Overall, the results from this study suggest that plants contribute directly to N_2O emission when NO_3^- is the N source but failed to identify the specific mechanism. However, this study also suggests that N_2O emission might be indirectly influenced by plant physiological changes induced by alterations in GS activity at different N rates. The Cadenza variety showed more efficient N assimilation with less N_2O emission, which could help reduce N_2O emissions in the field. A recent field study measuring N_2O emission from a wheat field reported that wheat genotypes with high N uptake potential contributed to reduced soil N_2O losses (Chen et al., 2021).

3.6 Conclusions

This study showed that the type and level of supplied N can affect the rate of N₂O emissions from wheat. Furthermore, N assimilation in wheat can vary between varieties under similar growth conditions. Applying KNO₃ slightly increased the shoot N₂O emission, while leaf N component concentrations were greater at high N levels and the shoot N₂O emission was higher at low N. The difference in leaf GS activity between Cadenza and Sokoll varieties may have contributed to variation in N₂O emission when KNO₃ was applied. The study suggests additional research to determine the relationship between plant N assimilation processes and gaseous N emissions and the potential for variety selection as a mitigation strategy for N₂O emissions.

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4 Effects of a Urease Inhibitor on Nitrogen Metabolism and Use Efficiency in Drought-stressed Wheat

Marieme Drame^{1,2*}, Maria Oszvald⁴, Alison Carswell¹, Jessica Hood⁵, William Roberts¹, Martin Jemo³, Mark Pawlett², Guy Kirk², Tom Misselbrook¹

¹Net Zero and Resilient Farming, Rothamsted Research, North Wyke, UK.

²Environment and Agrifood, Cranfield University, Cranfield, UK.

³AgroBiosciences Program, College of Agriculture and Environmental Sciences (CAES), Mohammed VI Polytechnic University (UM6P)

⁴Sustainable Soils and Crops, Rothamsted Research, Harpenden, UK.

⁵Intelligent Data Ecosystems, Rothamsted Research, Harpenden, UK

*Correspondence: marieme.drame@rothamsted.ac.uk

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4.1 Abstract

Nitrogen (N) fertiliser application can alleviate drought stress in crops. We studied the effects of a urease inhibitor (UI) applied with urea on N assimilation and use efficiency (NUE) in wheat under well-watered and drought-stressed conditions. Glasshouse pot experiments were conducted using three spring wheat varieties (Achtar, Cadenza, and Rihane) grown under well-watered and drought-stressed conditions at three N rates (0, 90, and 180 kg N ha⁻¹), with N applied as urea with and without the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT). Results showed drought-stress did not influence wheat leaf N assimilation; however, plant growth, biomass, grain weight, N uptake, and NUE were decreased. Application of the UI and N fertilisation at the rate of 180 kg ha⁻¹ increased leaf urea concentration, but did not improve wheat growth, grain weight, N uptake, or NUE, relative to urea alone. Nitrogen use efficiency was greater at 90 kg N ha⁻¹, with greater residual soil N at 180 kg N ha⁻¹. Of the three varieties, Cadenza was most tolerant of drought-stress with better growth, grain weight, N uptake, and NUE; Achtar recovered from drought-stress by developing additional tillers once watering was restarted, but yielded less than Cadenza but better than Rihane, the most drought-stress sensitive variety. The results demonstrate that NBPT can delay plant N uptake and

temporarily impair plant N assimilation with possible yield reduction. However, using N efficient varieties and applying UI treated urea at the right time can enhance NUE in wheat.

Keywords: Nitrogen metabolism, Urease inhibitor, Spring wheat, Drought tolerance, Urea fertiliser

4.2 Introduction

Drought stress affects wheat (*Triticum aestivum* L.) yields worldwide and is further exacerbated by climate change (Wang et al., 2016; Alam et al., 2021). Future climate change scenarios predict the most severe impacts in the much of Africa, Asia, and Central and South America (IPCC, 2023). Studies have demonstrated that crop productivity can be reduced by 50 to 73% under a limited water supply (Moradi-Ghahderijani et al., 2017; Azmat et al., 2020). Water deficit adversely affects many physiological and metabolic processes in plants (Kaya et al., 2006), impeding wheat performance at all growth stages (Farooq et al., 2014). Drought restricts plant photosynthesis, which causes oxidative stress in plant cells (induced by excess production of reactive oxygen species), damaging cell membranes, proteins and nucleic acids (Gou et al., 2017; Ru et al., 2023). These physiological drought-induced changes implicate nitrogen (N) metabolism as a critical process during plant water stress (Wang et al., 2017). Improved N uptake through N fertilisation has been reported to enhance plant tolerance to drought stress (Ru et al., 2023; Wang et al., 2017). The potential to attenuate drought stress can increase under high N supply (Iqbal et al., 2020; Wan et al., 2022). With increased N availability, plants may develop more extensive root systems, which allows them to access water from deeper soil layers during drought conditions (Wang et al., 2019). Additionally, N supply enhances the efficiency of photosynthesis by maintaining higher levels of chlorophyll content and improving other related processes (light absorption, stomatal conductance, CO₂ fixation), resulting in higher biomass and leaf area per unit of water used (Lawlor and Cornic, 2002). Nitrogen facilitates the production of osmolytes like proline and soluble sugars, which help maintain cell turgor pressure and prevent cellular dehydration during drought stress (Ashraf and Foolad, 2007). Nitrogen also promotes the synthesis of antioxidants and other stress-related proteins, which protect cells from oxidative damage caused by drought stress (Iqbal et al., 2020). These physiological and biochemical improvements

make plants more resilient to water stress, enabling them to sustain growth and yield in drought-prone environments. However, oversupplying crops with N fertiliser leads to significant N losses to the environment (Mahboob et al., 2023) as over 50% of the applied N is often unutilised by plants due to various factors including management, environmental and climatic conditions (Tilman et al., 2002).

Utilisation of N cycle inhibitors may offer an alternative way to augment fertiliser N use efficiency (NUE) and reduce N application rate (Mahboob et al., 2023). The inclusion of urease inhibitors (UI) with urea fertilisers effectively reduces ammonia emissions associated with urea applications (Sha et al., 2020; Drame et al., 2023). Urease inhibitors delay urea hydrolysis in soil, which may extend the period that urea remains available for plant uptake (Zanin et al., 2015). Consequently, using urea "protected" by UI is a viable tool for reducing ammonia emissions from agriculture (Sutton et al., 2022). However, previous studies have reported that the UI N-(n-butyl) thiophosphoric triamide (NBPT) can be absorbed by plant roots and inhibit urease activity in the plant, potentially disrupting plant N assimilation processes (Krogmeier et al., 1989; Artola et al., 2011; Cruchaga et al., 2011; Zanin et al., 2016).

The degree to which NBPT inhibits urease activity in plants varies depending on plant species and NBPT concentration, with less inhibition in species with high N assimilation capacity in leaves (Cruchaga et al., 2011) and at lower levels of NBPT (Artola et al., 2011). For example, NBPT induced an accumulation of urea and a reduction in amino acid and soluble protein content in pea and spinach leaves, with the effect being more pronounced in peas than in spinach (Cruchaga et al., 2011). Using ^{15}N derived from urea (24 h immersion of plant roots in treated solutions), Zanin et al. (2015) found that the inclusion of NBPT with urea significantly reduced N accumulation in plants (about 60% reduction) and impaired N translocation from the root to the shoot. According to Cruchaga et al. (2011), variations between plants (e.g., pea and spinach) could be due to differences in their absorption ability or urease enzyme structure.

Nonetheless, the impact of NBPT uptake with urea does not consistently impair plant development. Field and laboratory studies have reported improvements in yield, grain quality and NUE of crops receiving urea with NBPT (Wallace et al., 2020; Haque et al., 2021, 2022). Fertilisation with urea treated with NBPT increased maize yield and N

uptake relative to untreated urea in a highly weathered tropical soil (Mohd Zuki et al., 2020). Similarly, compared to urea alone, including NBPT with urea improved the yield and Cannabidiol content of Cannabis plant varieties (Kakabouki et al., 2021). Haque et al. (2021) measured significant increases in pineapple plant above-ground dry biomass, leaf area, N accumulation, partial factor productivity and average fruit weight when UI was included with urea compared to untreated urea.

Zanin et al. (2016) suggest that plants respond to their disrupted N metabolism, following absorption of NBPT, by activating compensation mechanisms, such as upregulation of metabolic processes associated with N-recycling, including phenylalanine and tyrosine. Haque et al. (2022) observed that foliar feeding of pineapple with urea, including NBPT, reduced malondialdehyde content, reducing drought-induced cell damage. Similarly, a study conducted by Gou et al. (2017) with maize plants grown in hydroponic solution also reported that urea treated with NBPT significantly improved drought tolerance of maize plants by protecting the photosynthetic apparatus, activating antioxidant defence systems, and improving osmoregulation. In summary, recent research findings suggest that including NBPT with urea fertiliser could increase plant resilience under different stress conditions; however, there is a lack of information on the effect of NBPT on wheat drought stress attenuation. Additionally, the number of studies reporting NBPT influence on plant N acquisition mechanisms is limited, with most existing studies consisting of short duration (e.g., two weeks or 24h exposure to NBPT in solution).

This study aimed to examine the efficacy of urea treated with NBPT to improve wheat NUE under well-watered and drought-stressed conditions and its potential to enhance the tolerance of specific wheat varieties to drought at different urea N application rates. The hypotheses were: 1) urea treated with UI NBPT improves wheat fertiliser NUE under well-watered and drought-stressed conditions, despite N assimilation impairments induced by UI in leaves; 2) wheat plants receiving urea treated with UI show greater resilience to drought stress.

4.3 Materials and Methods

4.3.1 Soil preparation and characterisation

A glasshouse study was conducted at Rothamsted Research, Harpenden in East England. A sandy loam soil collected to a depth of 10 cm from a bare-fallow treatment at the Woburn Experimental Farm (52°0' 50.73" N, 0°35' 5.63" W) in Bedfordshire was used for the experiments. This soil was selected due to its low nutrient supply status. The soil properties are included in Table 4-1. The mean annual precipitation and temperature at Woburn is 657.4 mm and 9.9°C (Evans et al., 2021). The collected soil was air-dried and passed through a 5 mm sieve before amending with 30% (weight basis) autoclaved perlite to improve its structure.

Table 4-1: Properties of the experimental soil

Property	Value
Soil type (FAO classification)	Arenosol
Sand, silt, clay content (%)	83, 10, 7
pH (1:2.5 soil:H ₂ O)	5.85 ± 0.139
Total soil C (g C kg ⁻¹ DW soil)	5.78 ± 0.002
Total soil N (g N kg ⁻¹ DW soil)	0.538 ± 0.010
Soil C:N ratio	10.7 ± 0.020
Nitrate + nitrite-N (mg N kg ⁻¹ DW soil; KCl soluble)	6.59 ± 0.731
Ammonium-N (mg N kg ⁻¹ DW soil; KCl soluble)	0.925 ± 0.109
Available P (mg P kg ⁻¹ DW soil; Mehlich III)	42.8 ± 0.117
Available K (mg K kg ⁻¹ DW soil; Mehlich III)	42.7 ± 0.239
Available Mg (mg Mg kg ⁻¹ DW soil; Mehlich III)	33.4 ± 0.683
Available Zn (mg Zn kg ⁻¹ DW soil; Mehlich III)	4.31 ± 0.081

DW is dry weight. Data are mean values ± standard error ($n = 4$, except total N and C ($n = 2$)). Mehlich III refers to the method of Mehlich (1984); Ammonium (NH₄⁺) and nitrite + nitrate N were measured after soil extraction with a 2 M KCl solution and analyzed using a Skalar SAN⁺ system (Skalar, UK). Total N and C were analyzed following dry combustion using a LECO TruMac combustion analyser (Stockport, UK).

4.3.2 Experimental design

Two glasshouse experiments were conducted simultaneously. Both experiments had a randomised complete block design with a factorial treatment structure. The fertiliser treatments were urea (U; Diamond Fertilisers, Lincolnshire, UK); urea with the urease inhibitor NBPT (treatment henceforth is US; Origin SUSTAIN®, Hertfordshire, UK) at 600 ppm; and a zero N control (C). Two fertiliser rates, 90 kg N ha⁻¹ and 180 kg N ha⁻¹, were applied in a split application. The N rates used were based on the UK nutrient management guide (RB209) soil nitrogen supply index, taking account of the low soil residual N. The N rates are within the range of those that would be applied for wheat production in temperate and Mediterranean agriculture. The watering regime included well-watered (WW) and drought-stressed (DS) conditions, which corresponded to maintaining the soil at 75 and 30% of soil water holding capacity (WHC), respectively. Three commonly grown spring wheat varieties were used in this experiment for their contrast in production area, which were the Cadenza variety from UK, and the Rihane and Achtar varieties from Morocco experiencing recurrent drought with a decrease in

average rainfall (Devkota et al., 2023). Treatments were replicated 5 times, giving a total of 150 experimental units in each experiment. In Experiment 1, plant responses were assessed at the molecular-physiological level, which involved destructive sampling of the fresh plants at different growth stages, whereas in Experiment 2 plants were grown through to harvest to determine fertiliser NUE.

4.3.3 Plant growth conditions and fertilisation

Wheat seeds were prepared by sterilising with 100% ethanol for 2 minutes then treating with 5% sodium hypochlorite for 15 minutes followed by successive washing with sterile water. Seeds were soaked in sterilized water overnight at 4°C in the dark prior to pre-germinating on wet filter paper for 7 days at room temperature. Square pots (90 × 90 × 100 mm; length × width × depth) were packed with 600 g of the prepared soil. For Experiment 1, three seedlings of similar size were transplanted per pot, for 2 destructive sampling events. For Experiment 2, one seedling was transplanted per pot to develop to maturity. Plants were grown at 24°C/21°C (day/night) with 16 h light and 8 h darkness. Supplementary light was supplied by Kropstek LED light with a pink spectrum to keep the lighting above 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. All plants were WW at the start of the experiment. The DS treatment was introduced at the start of stem elongation by withholding water supply for about 2 days until soil moisture dropped below 30% WHC. Thereafter, soil moisture was readjusted and maintained at 30% WHC. Soil moisture content was checked daily with a moisture meter (Delta-T Devices Ltd. Cambridge, UK) and water added as required to keep the soil at the desired WHC. Pots under the WW treatment were maintained at 75% WHC throughout.

Granulated urea fertiliser was surface applied as two split applications per the RB209 recommendation for spring sown wheat in Experiment 1 as a pre-plant application and at the stem elongation stage (a day before starting the DS; Table 4-2). Experiment 2 received a third application at the grain filling stage (Table 4-2). Potassium and phosphorus were applied as a basal application at 30 and 40 kg ha⁻¹ of P and K, respectively. Micronutrients were applied with a modified Letcombe solution (Drew and Saker, 1984).

Table 4-2: Nitrogen fertiliser rates and application timings

Fertiliser rate (kg N ha ⁻¹)	Basal	Stem elongation	Grain filling
Experiment 1			
90	40	50	-
180	40	140	-
Experiment 2			
90	20	50	20
180	40	100	40

4.3.4 Plant and soil sampling and analysis

Experiment 1:

Fresh wheat leaves were sampled from a single plant in each pot five days after the second N application, corresponding to the fourth day of DS at 30% WHC. A second sampling was conducted from a second plant in each pot corresponding to thirteen days of DS at 30% WHC. The two upper, fully expanded leaves were sampled, frozen immediately in liquid N and stored at -80°C prior to analysis. The remaining above-ground biomass was cut at 1 cm height above the soil surface and dried at 80°C for 24 h to determine plant dry matter at the time of sampling. The frozen leaf tissues were finely ground in liquid N and tissue ammonium (NH₄⁺), urea, protein and glutamine synthetase (GS) activity determined using the following colorimetric methods with a microplate spectrophotometer (SPECTRAmax® 340PC; Molecular Devices Corporation, California, USA). A 100 mg fresh leaf sample was mixed with 1 ml of 0.05 M sulfuric acid and incubated at room temperature for 1 h (Mattsson and Schjoerring, 1996) and then centrifuged at 13,000 *g* for 15 min. The supernatant was sampled and used to measure both leaf NH₄⁺ and urea concentration using the Megazyme rapid ammonia and urea/ammonia kit (K-AMIAR 02/21). Protein concentration was determined following

the method of Bradford et al. (1976), which involved binding of Coomassie Brilliant Blue G-250 to proteins. For GS activity, a 100 mg sample of fresh leaf tissue was mixed with 1 ml of buffer solution (containing 25 mM TRIS–HCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Dithiothreitol, 1 mM Magnesium Chloride, 0.2 g polyvinyl pyrrolidone and 2 mM leupeptin, pH 7.6 (Oszvald et al., 2022). GS activity was then measured according to O’Neal and Joy, (1973) by incubating 50 µl leaf extract, extracted with an assay medium containing 600 mM glutamate, 45 mM hydroxylamine hydrochloride, 150 mM magnesium sulphate heptahydrate, 30 mM EDTA disodium salt, and 60 mM adenosine triphosphate for 30 min at 30°C in a shaker incubator. The enzyme reaction was terminated with 300 µl of stopping solution (0.67M FeCl₃, 0.2M TCA, 0.7M HCl).

Experiment 2:

Plant tiller number was determined at the grain filling stage, and whole plant dry weight and grain weight were quantified at harvest. Total N was measured in the grain and the rest of plant aboveground biomass using the LECO TruMac combustion analyser. After harvest, soil from each pot was cleared of plant roots and soil subsamples were milled prior to determination of total N concentration.

4.3.5 Wheat N uptake, N use efficiency and N balance

Wheat aboveground biomass (straw) and grain N uptake were estimated by multiplying the straw and grain weights by the corresponding total N concentrations. The wheat total N uptake refers to the sum of the respective straw and grain N.

Fertiliser NUE was evaluated using the following indices described by Dobermann (2007); however, in this study indices were determined for the whole plant production (biomass and grain) rather than grain alone. Apparent crop N recovery efficiency (RE_N , %) was calculated as:

$$RE_N = \frac{N_F - N_C}{N_R} \times 100 \quad (4-1)$$

where, N_F and N_C are total N uptake of whole plant from fertilised and control plots (kg ha⁻¹), respectively, and N_R is the rate of N applied (kg N ha⁻¹). Physiological N efficiency (PE_N , kg kg⁻¹) was measured as:

$$PE_N = \frac{W_F - W_C}{N_F - N_C} \quad (4-2)$$

where W_F and W_C are whole plant weight from N fertilised and control plots (kg), respectively. Agronomic N efficiency (AE_N , kg kg⁻¹) was calculated as:

$$AE_N = \frac{W_F - W_C}{N_R} \quad (4-3)$$

Internal N utilization efficiency (IE_N , kg kg⁻¹) was calculated as:

$$IE_N = \frac{W_F}{N_F} \quad (4-4)$$

Nitrogen balance ($N_{Balance}$) was calculated according to Sainju et al. (2017) as:

$$N_{Balance} = N_{Inputs} - N_{Outputs} - \Delta N \quad (4-5)$$

where N_{Inputs} = N fertiliser applied + soil residual N, $N_{Outputs}$ = total N uptake (straw and grain), and ΔN is the change in soil total N (or residual N), determined as the difference between soil total N content after harvest and at the start of the experiment.

4.3.6 Data analysis

Treatment effects on plant growth and yield parameters, leaf N components and NUE indices were evaluated using a linear mixed model with random models specifying the design structure of trays nested within replicates (Replicate/Tray) and a fixed model defining the interaction between each treatment, specifically: Variety*Water regime*(Fertilisation/(Fertiliser type*Fertiliser rate)). Models were estimated using REML with F-statistics obtained using the Kenward-Roger denominator degrees of freedom. The tiller number was analysed using a generalised linear mixed model with the same random and fixed model structure as described above. Pairwise comparisons of the data were made using the least significant difference test, with differences between

treatments considered significant where $p < 0.05$. Wheat tiller number, leaf urea and protein content, and AE_N required log transformation to meet model assumptions about normal distribution of data. In contrast, leaf GS activity, NH_4^+ concentration and dry biomass in Experiment 1 were transformed using a square root transformation to meet model assumptions of data normality. Pearson correlation analyses performed for leaf N components and NUE indices and all statistical analyses were carried out using GenStat (22nd edition).

4.4 Results

4.4.1 Experiment 1: Wheat leaf N components

Watering regime

Leaf N components were not significantly influenced by the watering regime, with NH_4^+ , urea and protein concentrations and GS activity being statistically similar between the WW and DS treatments for both sampling events. However, dry biomass yield was affected by the watering regime in sampling event 2, with significantly greater biomass produced under the WW condition (Table 4-3).

Wheat variety

At sampling event 1, leaf NH_4^+ concentration was statistically similar for all wheat varieties. Whereas leaf urea concentration was significantly greater in Cadenza leaves compared to Achar, with that for Rihane being intermediate (Table 4-3). Similarly, leaf GS activity was significantly greater in Cadenza and Rihane, relative to Achar. Additionally, Cadenza and Rihane had significantly greater dry biomass than Achar.

At sampling event 2, leaf NH_4^+ was significantly greater in Rihane compared to Achar and Cadenza, whereas leaf urea concentration in both Cadenza and Rihane was significantly greater than in Achar (Table 4-3). Leaf GS activity in Rihane was significantly greater than in Cadenza, with Achar being intermediate. Dry biomass was significantly greatest in Cadenza, with Rihane and Achar similar to each other. There was no significant difference between varieties in leaf protein at either sampling event.

Table 4-3: Wheat leaf N components at the first and second sampling events according to linear mixed effects model in response to variety, water regime and N source.

	NH ₄ ⁺ S1 (μmol g ⁻¹)	NH ₄ ⁺ S2 (μmol g ⁻¹)	Urea S1 (μmol g ⁻¹)	Urea S2 (μmol g ⁻¹)	GS S1 (μmol GHA mg FW ⁻¹ h ⁻¹)	GS S2 (μmol GHA mg FW ⁻¹ h ⁻¹)	Protein S1 (mg g ⁻¹)	Protein S2 (mg g ⁻¹)	Dry biomass S1 (g)	Dry biomass S2 (g)
Variety										
Achtar	1.33±0.067a	1.51±0.081a	-0.680±0.151a	-1.71±0.164a	12.9±0.424a	15.5±0.220ab	0.268±0.042a	0.710±0.043a	0.550±0.018a	0.678±0.023a
Cadenza	1.42±0.063a	1.50±0.081a	-0.234±0.140b	-0.864±0.170b	14.8±0.437b	15.4±0.226a	0.388±0.043a	0.745±0.043a	0.715±0.018c	0.851±0.022b
Rihane	1.23±0.075a	1.72±0.085b	-0.421±0.168ab	-1.03±0.183b	14.7±0.543b	16.2±0.271b	0.315±0.047a	0.809±0.050a	0.601±0.018b	0.707±0.024a
Water regime										
DS	1.30±0.057a	1.53±0.071a	-0.420±0.133a	-1.26±0.138a	14.2±0.366a	15.8±0.199a	0.295±0.038a	0.774±0.037a	0.628±0.016a	0.702±0.019a
WW	1.35±0.048a	1.62±0.074a	-0.470±0.117a	-1.14±0.144a	14.1±0.393a	15.6±0.194a	0.351±0.038a	0.735±0.038a	0.615±0.016a	0.788±0.019b
Fertilisation										
Unfertilised	1.13±0.122a	1.61±0.100a	-1.79±0.256a	-1.68±0.225a	10.8±0.880a	12.8±0.362a	0.247±0.074a	0.539±0.069a	0.396±0.022a	0.469±0.029a
Fertilised	1.37±0.031b	1.57±0.064a	-0.108±0.090b	-1.08±0.111b	14.8±0.269b	16.4±0.149b	0.340±0.031a	0.809±0.028b	0.678±0.014b	0.814±0.016b
Fertiliser type										
C	1.13±0.122a	1.61±0.0100a	-1.79±0.256a	-1.68±0.225a	10.8±0.880a	12.8±0.362a	0.247±0.074a	0.539±0.069a	0.396±0.022a	0.469±0.029a
U	1.33±0.055a	1.55±0.077a	-1.09±0.129b	-1.77±0.157a	14.9±0.377b	16.6±0.211b	0.300±0.038a	0.807±0.040a	0.710±0.017c	0.810±0.021b
US	1.42±0.053a	1.58±0.078a	0.879±0.126c	-0.395±0.157b	14.7±0.383b	16.2±0.208b	0.379±0.039a	0.810±0.039a	0.646±0.017b	0.819±0.021b
Fertiliser rate (kg N ha ⁻¹)										
0	1.13±0.122a	1.61±0.0100a	-1.79±0.256a	-1.68±0.225a	10.8±0.880a	12.8±0.362a	0.247±0.074a	0.539±0.069a	0.396±0.022a	0.469±0.029a
90	1.32±0.054a	1.56±0.078a	-0.710±0.129b	-1.70±0.160a	14.6±0.387b	16.2±0.203b	0.312±0.038a	0.817±0.038a	0.671±0.017b	0.839±0.020b
180	1.42±0.053a	1.57±0.077a	0.495±0.126c	-0.459±0.155b	15.0±0.373b	16.6±0.215b	0.367±0.038a	0.800±0.041a	0.685±0.017b	0.790±0.021b
Significance of fixed effects										
Variety	0.232	0.035	0.042	<0.001	<0.001	0.049	0.080	0.328	<0.001	<0.001
Water regime	0.333	0.281	0.803	0.846	0.901	0.482	0.216	0.898	0.544	<0.001
Fertilisation	0.043	0.678	<0.001	0.012	<0.001	<0.001	0.177	<0.001	<0.001	<0.001

S1 and S2 refer to the 1st and 2nd plant leaf sampling. Values are predicted mean values ± standard error (n = 5) from REML analysis. Letters depict significant difference between means within columns (p < 0.05).

N fertiliser rate and type

Fertilisation with N increased leaf NH_4^+ (in sampling event 1), urea, GS activity, protein (in sampling event 2), and dry biomass (Table 4-3). At sampling event 1, leaf NH_4^+ was significantly greater under the US treatment at the highest N rate of 180 kg N ha^{-1} compared to 90 kg N ha^{-1} and relative to the U treatment at 180 kg N ha^{-1} (Figure 4-1a). Leaf urea was greater for US treated plants and at 180 kg N ha^{-1} . Leaf protein concentration was not influenced by N fertiliser type and rate at sampling event 1. However, dry biomass was greater under U than US (Table 4-3).

At sampling event 2, leaf NH_4^+ was similar between fertilised and unfertilised plants. Leaf urea concentration was significantly greater under US than under U at both sampling events (Figure 4-2a, b) and, under US, greater for the 180 kg N ha^{-1} application rate treatment.

Overall, leaf N components (NH_4^+ , protein, GS activity) and dry biomass significantly increased between sampling events 1 and 2, excluding leaf urea concentration, which declined over this time, particularly at lower N rate (Table 4-3).

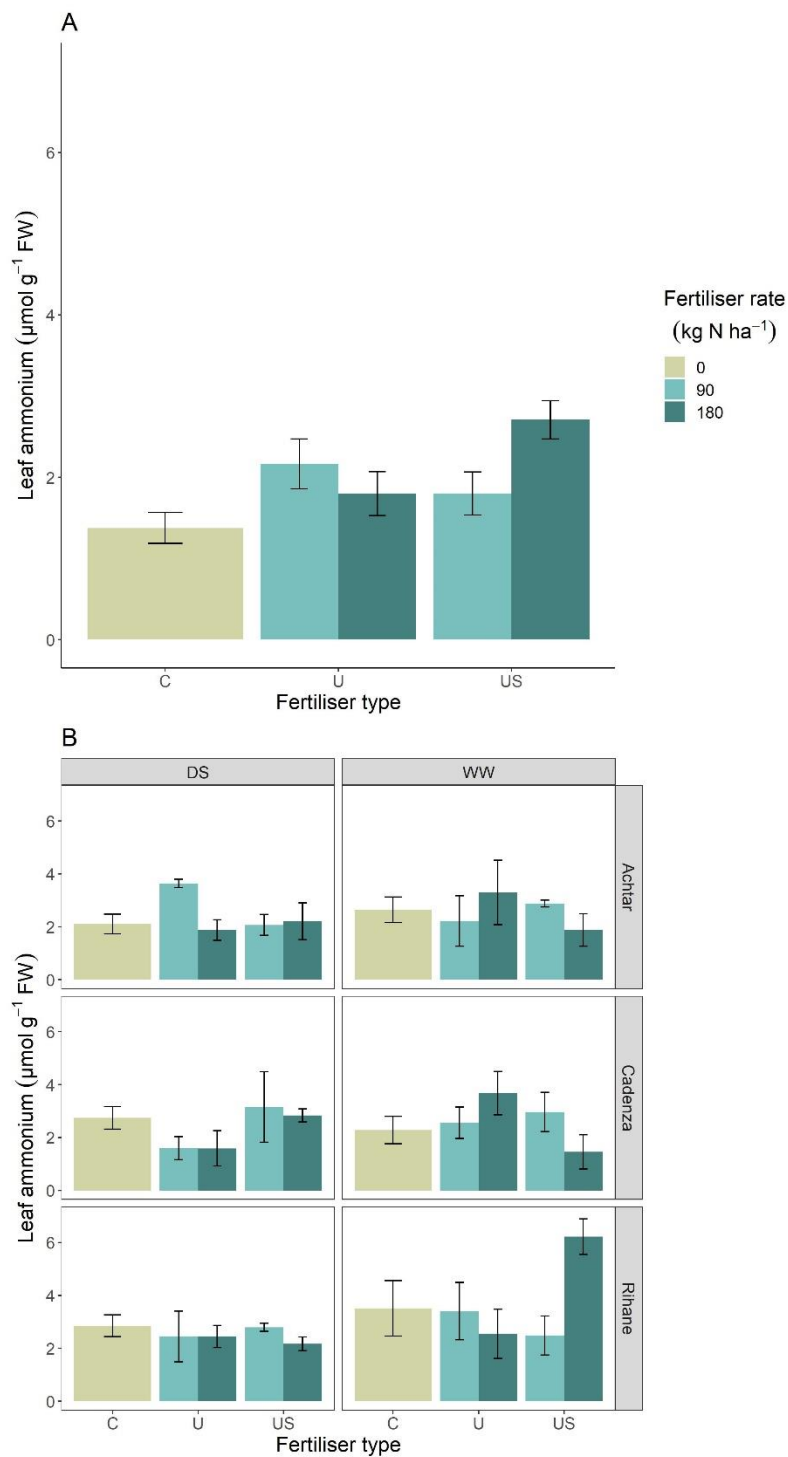


Figure 4-1: Leaf NH_4^+ concentrations as influenced by (a) fertiliser type, averaged over fertiliser rate at the first sampling event 1; and (b) variety, water regime and N fertiliser rate, averaged over fertiliser types at the second sampling event. Data are means $\pm$ standard errors ($n = 5$). Letters depict significant difference between means ($p < 0.05$).

Wheat variety, watering regime and fertiliser interactions

At sampling event 1, leaf urea concentration was significantly greater at the highest N rate of 180 compared to 90 kg N ha⁻¹ (Figure 4-2c), and significantly greater in US compared to U fertilised wheat, particularly for Cadenza and Rihane (Figure 4-2d). For leaf protein concentration, there was a significant difference between N fertilised and unfertilised plants under WW, but this was not the case under DS (Figure 4-3a). Dry biomass was influenced by the combined effect of variety, fertiliser type and fertiliser rate (Supplementary information, Table D-1) with Achtar having significantly greater dry biomass under U than US fertilised plants at 180 kg N ha⁻¹, whereas for Rihane this was observed at 90 kg N ha⁻¹ (Figure 4-3b).

Additionally, under DS for all varieties, there was no significant difference in GS activity between U and US fertilisers (Figure 4-3c). Under WW condition, leaf GS activity in Rihane was significantly lower under US compared to U treatment. Furthermore, the interaction between watering regime, fertiliser type and rate was significant for leaf NH₄⁺. This interaction varied significantly between varieties ($p = 0.003$; Figure 4-1b) at sampling event 2, with significant differences observed under WW condition, with the greatest effects observed in Rihane. Under WW conditions, Rihane leaf NH₄⁺ concentration was significantly higher under US than under U fertilised plants at the 180 kg N ha⁻¹ rate; and leaf NH₄⁺ greater at 180 than 90 kg N ha⁻¹ for US treated plants (Figure 4-1b). The correlation analysis of leaf N components at sampling events 1 and 2 revealed a significant positive relationship between GS activity and dry biomass. Similarly, leaf urea and NH₄⁺ concentrations were significantly positively correlated at sampling events 1 and 2 (Supplementary information, Table D-4 and D-5).

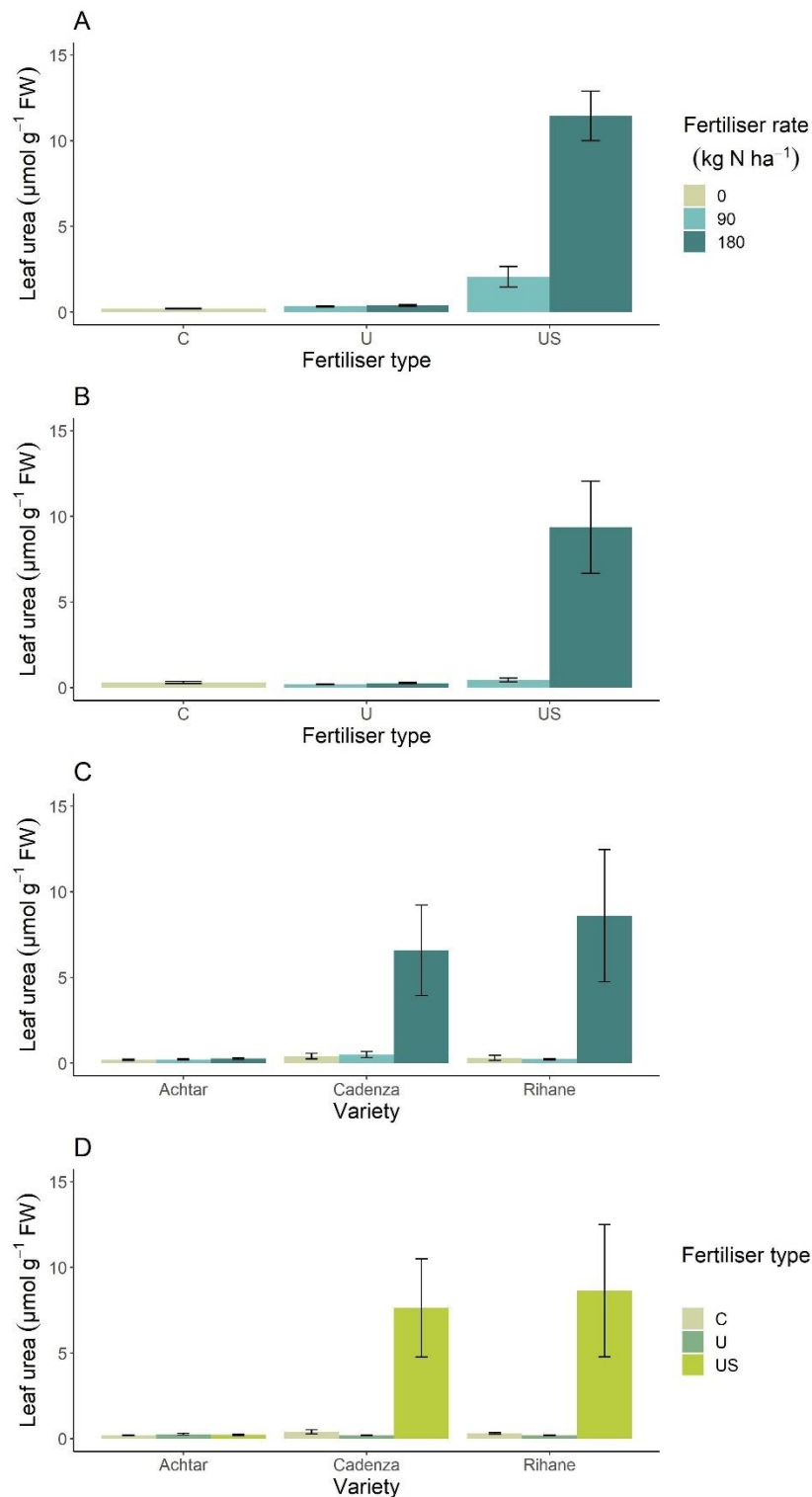


Figure 4-2: Wheat leaf urea concentration as influenced by (a) fertiliser type, averaged over fertiliser rate at the first sampling event; (b) fertiliser type, averaged over fertiliser rate at the second sampling event; (c) wheat variety, averaged over fertiliser rate at the second sampling event; and (d) wheat variety, averaged over fertiliser type at the second sampling event. Data are means $\pm$ standard errors ($n = 5$). Letters depict significant difference between means ($p < 0.05$).

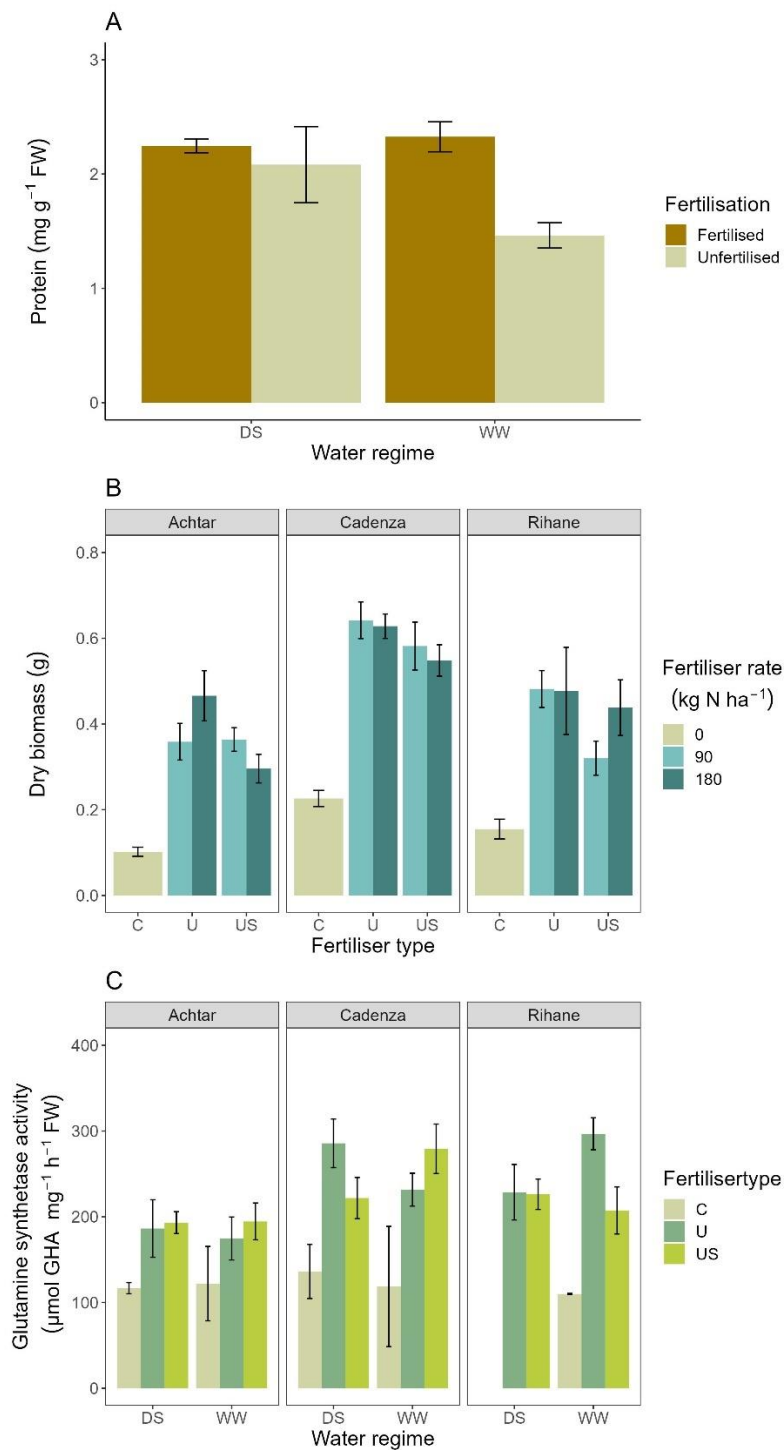


Figure 4-3: (a) Leaf protein content of wheat varieties supplied with N fertiliser under the two water regimes at the second sampling event; (b) Wheat dry biomass as influenced by fertiliser rate, averaged across variety and fertiliser type at the first leaf sampling event; and (c) glutamine synthetase activity of wheat varieties supplied with different fertiliser types under the two water regimes at the first leaf sampling event. Data are means $\pm$ standard errors ($n = 5$). Letters depict significant difference between means ($p < 0.05$).

4.4.2 Experiment 2: Wheat growth parameters

Watering regime

Except for tiller number at harvest, all other measured plant growth and yield parameters were significantly influenced by watering regime ($p < 0.001$, Table 4-4) with the greatest yield parameters observed under WW condition.

Wheat variety

Tiller number (during drought and at harvest), dry biomass and grain weight varied significantly between wheat varieties ($p < 0.001$, Table 4-4). In general, Cadenza performed better for these yield parameters than either Achtar or Rihane. Tiller number during DS was not significantly different between Achtar and Rihane, whereas after drought and by the time of harvest, Achtar developed significantly more tillers than Rihane. Final dry biomass and grain weight were significantly greater in Achtar than Rihane (Table 4-4).

N fertiliser

Under unfertilised growth conditions, there were no significant differences between the three varieties in all determined parameters. Similarly, there were no significant differences between WW and DS under unfertilised conditions, in tiller number developed during the drought imposed on the DS treatment (Figure 4-4a), or in grain weight at harvest (Figure 4-5a). The application of N fertiliser to plants promoted plant growth and grain production under both DS and WW conditions compared to unfertilised plants independent of fertiliser type or rate ($p < 0.001$). Wheat dry biomass under U was significantly greater than under US, and plants receiving 180 kg N ha⁻¹ had greater dry biomass compared to those receiving 90 kg N ha⁻¹ (Table 4-4). Wheat grain weight was significantly affected by fertiliser type and rate, with greater values under U compared to US at the 180 kg N ha⁻¹ N rate (Figure 4-5b).

Table 4-4: Plant growth parameters, yield and N uptake according to linear mixed effects model in response to variety, water regime, and nitrogen source.

		Tiller number during drought	Tiller number at harvest	Plant biomass (g)	Grain weight (g)	Total N uptake (g)
Variety						
	Achtar	0.441±0.079 ^a	0.636±0.065 ^b	1.85±0.112 ^b	1.35±0.133 ^b	0.065±0.003 ^b
	Cadenza	0.762±0.073 ^b	0.918±0.060 ^c	2.00±0.110 ^b	1.70±0.131 ^c	0.076±0.003 ^c
	Rihane	0.400 ±0.078 ^a	0.449±0.068 ^a	1.03±0.111 ^a	1.09±0.131 ^a	0.044±0.003 ^a
Water regime						
	DS	0.354±0.073 ^a	0.615±0.058 ^a	1.46±0.100 ^a	0.989±0.124 ^a	0.055±0.003 ^a
	WW	0.714±0.069 ^b	0.720±0.056 ^a	1.80±0.100 ^b	1.78±0.124 ^b	0.068±0.003 ^b
Fertilisation						
	Unfertilised	-0.006±0.097 ^a	-0.002±0.093 ^a	0.491±0.144 ^a	0.491±0.144 ^a	0.016±0.004 ^a
	Fertilised	0.669±0.066 ^b	0.835±0.050 ^b	1.60±0.118 ^b	1.60±0.118 ^b	0.073±0.002 ^b
Fertiliser type						
	C	-0.006±0.097 ^a	-0.002±0.093 ^a	0.492±0.127 ^a	0.491±0.144 ^a	0.016±0.004 ^a
	U	0.685±0.072 ^b	0.855±0.058 ^b	2.03±0.106 ^c	1.67±0.128 ^b	0.066±0.003 ^b
	US	0.654±0.073 ^b	0.815±0.058 ^b	1.80±0.106 ^b	1.54±0.128 ^b	0.080±0.003 ^b
Fertiliser rate (kg N ha ⁻¹)						
	0	-0.006±0.097 ^a	-0.002±0.093 ^a	0.492±0.127 ^a	0.491±0.144 ^a	0.016±0.004 ^a
	90	0.652±0.073 ^b	0.793±0.059 ^b	1.80±0.106 ^b	1.60±0.128 ^b	0.076±0.003 ^b
	180	0.686±0.072 ^b	0.877±0.057 ^b	2.02±0.106 ^c	1.61±0.128 ^b	0.070±0.003 ^c
<i>Significance of fixed effects</i>						
Variety		<0.001	<0.001	<0.001	<0.001	<0.001
Water regime		<0.001	0.236	<0.001	<0.001	<0.001
Fertilisation		<0.001	<0.001	<0.001	<0.001	<0.001

Values are predicted mean values ± standard error (n = 5) from GLMM and REML analysis. Letters depict significant difference between means within columns (p < 0.05).

Variety, water regime and N fertiliser interactions

During DS there was a significant interaction between variety and watering regime ($p = 0.028$, Supplementary information Table D-2) in tiller number, with more tillers under WW than DS for all three varieties. Under WW and DS, Cadenza had more tillers than both Achtar and Rihane, which were statistically similar (Figure 4-4b). However, after the DS was removed and up to harvest, the tiller number in Cadenza was not different between WW and DS. In contrast, for Achtar, the tiller number under DS became significantly greater than WW under the U treatment. In contrast, for Rihane, the tiller number under WW was still higher than under DS (Figure 4-4c). For US fertilised plants, there was no significant difference in tiller number between WW and DS for the three varieties. Achtar was the only variety significantly affected by fertiliser type, with U having a greater tiller number than US under DS (Figure 4-4c). The fertiliser rate did not influence the tiller number in this study.

The interacting effects of variety, watering regime and fertiliser rate significantly influenced wheat grain weight ($p = 0.012$). Grain weight was significantly greater under WW than DS conditions at both applied N rates and all varieties, except for Achtar at 180 kg N ha⁻¹. For each variety under WW and DS, there was no significant effect of N rate on grain weight (Figure 4-5c), except for Achtar under DS, where grain weight was significantly greater at 180 kg N ha⁻¹ compared to 90 kg N ha⁻¹.

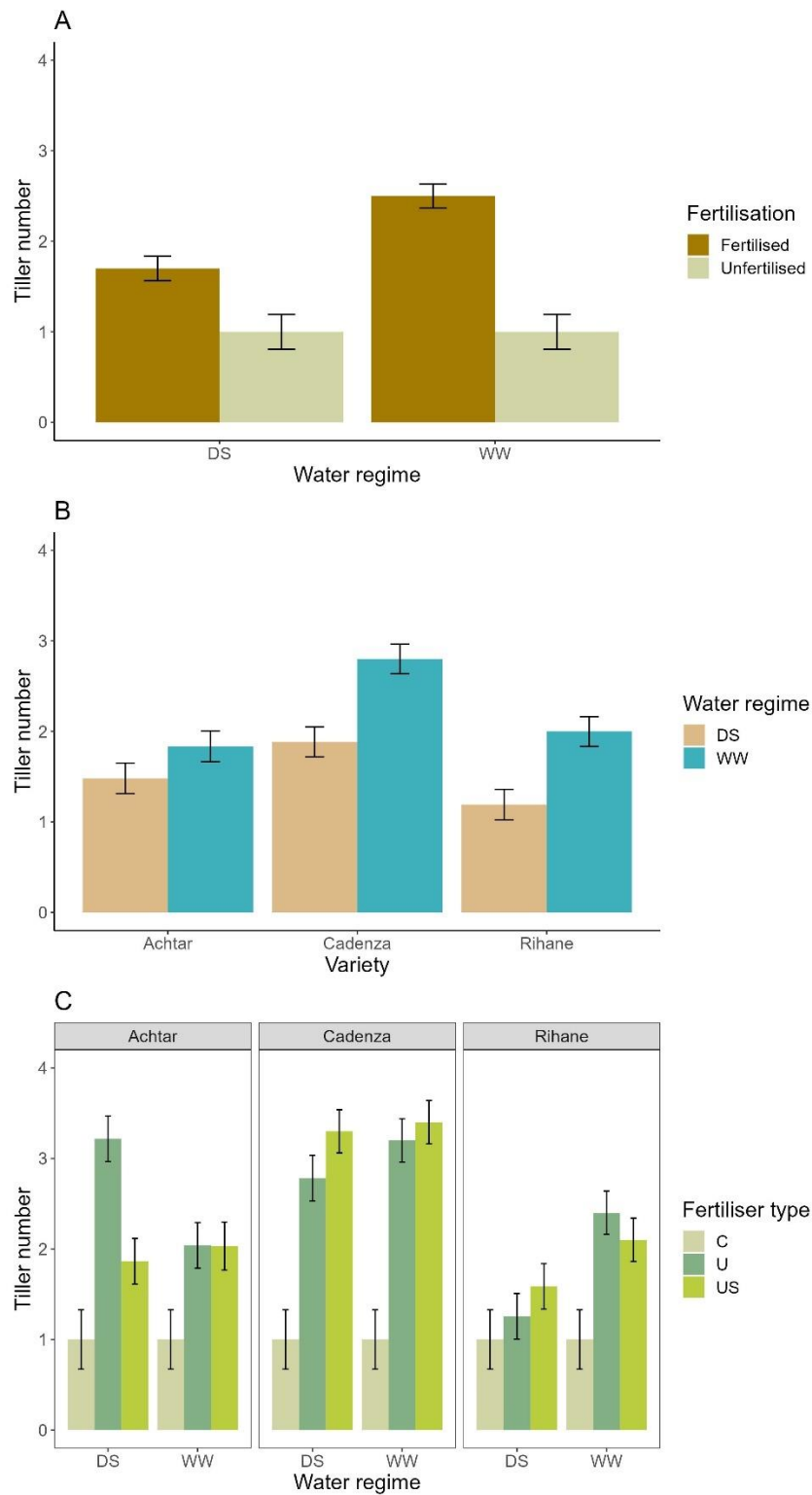


Figure 4-4: Tiller number as influenced by (a) water regime, averaged over fertilisation condition; (b) wheat variety, averaged over water regime during drought stress; and (c) wheat variety and water regime, averaged over fertiliser type at harvest. Data are means $\pm$ standard errors ($n = 5$). Letters depict significant difference between means ($p < 0.05$).

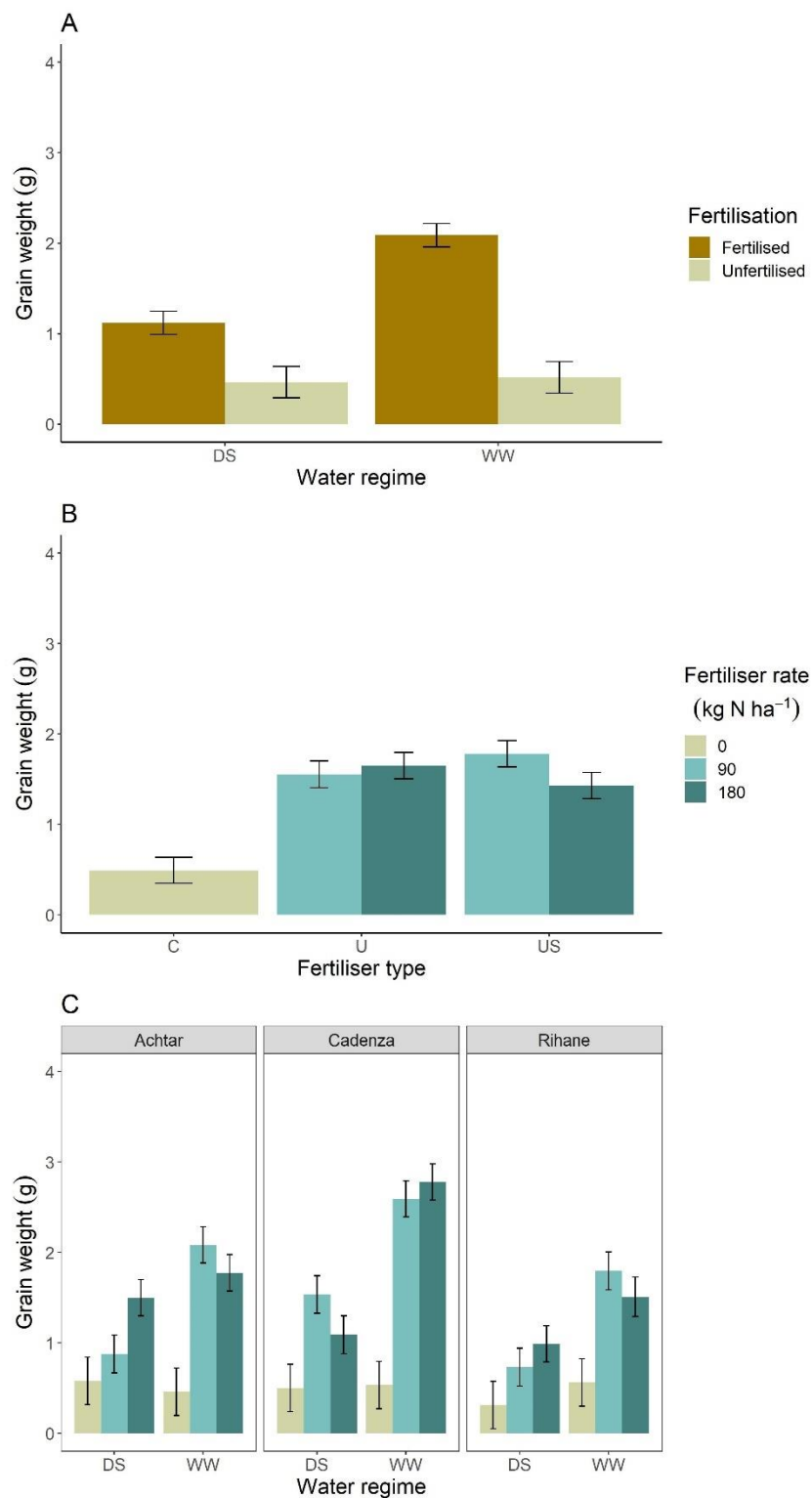


Figure 4-5: Grain weight as influenced by (a) water regime, averaged over fertilisation condition; (b) fertiliser type, averaged over fertiliser rate; and (c) variety and water regime, averaged over fertiliser rate. Data are means $\pm$ standard errors ($n = 5$). Letters depict significant difference between means ($p < 0.05$).

4.4.3 Nitrogen uptake and use efficiency

Watering regime

Well-watered plants had significantly more N uptake than DS plants. Similarly, the watering regime significantly affected all NUE indices, which were greater under WW (Table 4-5).

Wheat variety

Wheat variety had a significant effect on total N uptake (in grain and above-ground biomass) at harvest ($p < 0.001$), which was greatest for Cadenza, followed by Achar and then Rihane (Table 4-4). The NUE indices RE_N and AE_N were significantly affected by variety ($p < 0.001$; Table 4-5). Cadenza had higher RE_N followed by Achar, then Rihane. However, for AE_N , Cadenza and Achar were not significantly different, but both were significantly greater than Rihane.

N Fertiliser

Nitrogen uptake was significantly greater for N-fertilised plants than unfertilised ones, and N uptake was generally greater at 180 kg N ha⁻¹ compared to 90 kg N ha⁻¹. In contrast, fertiliser type had no significant effect on nitrogen uptake (Table 4-4). Fertiliser type did not significantly impact NUE indices in this study, however, NUE indices were significantly higher at 90 compared to 180 kg N ha⁻¹.

Table 4-5: Nitrogen use efficiency indices and balance according to linear mixed model effects in response to variety, water regime, and nitrogen source.

	Apparent recovery efficiency (%)	Physiological efficiency (g g ⁻¹)	Agronomic efficiency (g g ⁻¹)	Internal utilisation efficiency (g g ⁻¹)	Nitrogen balance
Variety					
Achtar	56.8±3.41 ^b	44.3±2.97 ^a	3.12±0.103 ^b	48.9±2.42 ^a	0.30±0.010 ^a
Cadenza	67.3±3.23 ^c	44.8±2.87 ^a	3.30±0.098 ^b	48.1±2.38 ^a	0.30±0.010 ^a
Rihane	40.8±3.46 ^a	46.0±3.00 ^a	2.61±0.104 ^a	45.8±2.39 ^a	0.32±0.010 ^b
Water regime					
DS	46.7±2.94 ^a	41.0±2.72 ^a	2.67±0.091 ^a	43.3±2.27 ^a	0.32±0.009 ^b
WW	63.3±3.00 ^b	49.2±2.75 ^b	3.34±0.092 ^b	51.9±2.26 ^b	0.30±0.009 ^a
Fertiliser type					
U	55.2±2.94 ^a	46.3±2.72 ^a	3.04±0.091 ^a	48.0±2.27 ^a	0.31±0.009 ^a
US	54.8±3.00 ^a	43.8±2.75 ^a	2.97±0.092 ^a	47.2±2.26 ^a	0.31±0.009 ^a
Fertiliser rate (kg N ha ⁻¹)					
90	67.3±2.97 ^b	48.1±2.73 ^b	3.28±0.091 ^b	50.4±2.27 ^b	0.29±0.009 ^a
180	42.7±2.98 ^a	42.0±2.74 ^a	2.73±0.091 ^a	44.8±2.26 ^a	0.33±0.009 ^b
<i>Significance of fixed effects</i>					
Variety	<0.001	0.795	<0.001	0.289	0.044
Water regime	<0.001	<0.001	<0.001	<0.001	0.003
Fertiliser type	0.920	0.329	0.624	0.622	0.529
Fertiliser rate	<0.001	0.005	<0.001	<0.001	<0.001

Values are predicted mean values ± standard error (n = 5) from REML analysis. Letters depict significant difference between means within columns (p < 0.05).

Variety, watering regime and fertiliser interactions

The watering regime and the N application rate in N fertilised pots significantly affected all NUE indices ($p < 0.001$), with RE_N , PE_N , AE_N and IE_N significantly greater under WW compared with DS and greater at the lower N application rate. There was a three-way interactive effect of variety, water regime and fertiliser on N uptake and AE_N ($p = 0.048$ and $p = 0.043$, respectively, supplementary information Table D-3). Under DS, N uptake was significantly greater at 180 kg N ha⁻¹ for Achtar only, while under WW, N uptake was significantly greater at 180 kg N ha⁻¹ for Cadenza (Figure 4-6a). For Cadenza, N uptake was significantly increased under WW compared to DS at 180 kg N ha⁻¹, whereas for Rihane the same effect was observed at 90 kg N ha⁻¹. Nitrogen uptake between WW and DS was not significantly different in Achtar at either N rate. Under DS, AE_N was significantly greater at 90 kg N ha⁻¹ for Cadenza only. Under WW, AE_N was significantly greater at 90 kg N ha⁻¹ for all three varieties. At 90 and 180 kg N ha⁻¹, AE_N increased significantly under WW compared to DS, except for Achtar, where DS and WW were not different at the 180 kg N ha⁻¹ rate (Figure 4-6b). There were significant positive relationships between NUE indices, particularly between AEN and REN , PEN , and IE_N as well as IE_N and both REN and PEN (Supplementary information, Table D-6).

4.4.4 Nitrogen balance

Nitrogen balance was positive in this study and was significantly affected by the main effects of variety ($p = 0.044$), water regime ($p = 0.003$) and fertiliser rate ($p < 0.001$; Table 4-5). Residual soil N at the end of the experiment was greater for Rihane than Cadenza or Achtar (Table 4-5). Additionally, residual soil N was greater under DS compared to WW. Similarly, in fertilised pots, residual soil N was greater at the 180 kg N ha⁻¹ rate compared to the 90 kg N ha⁻¹ rate.

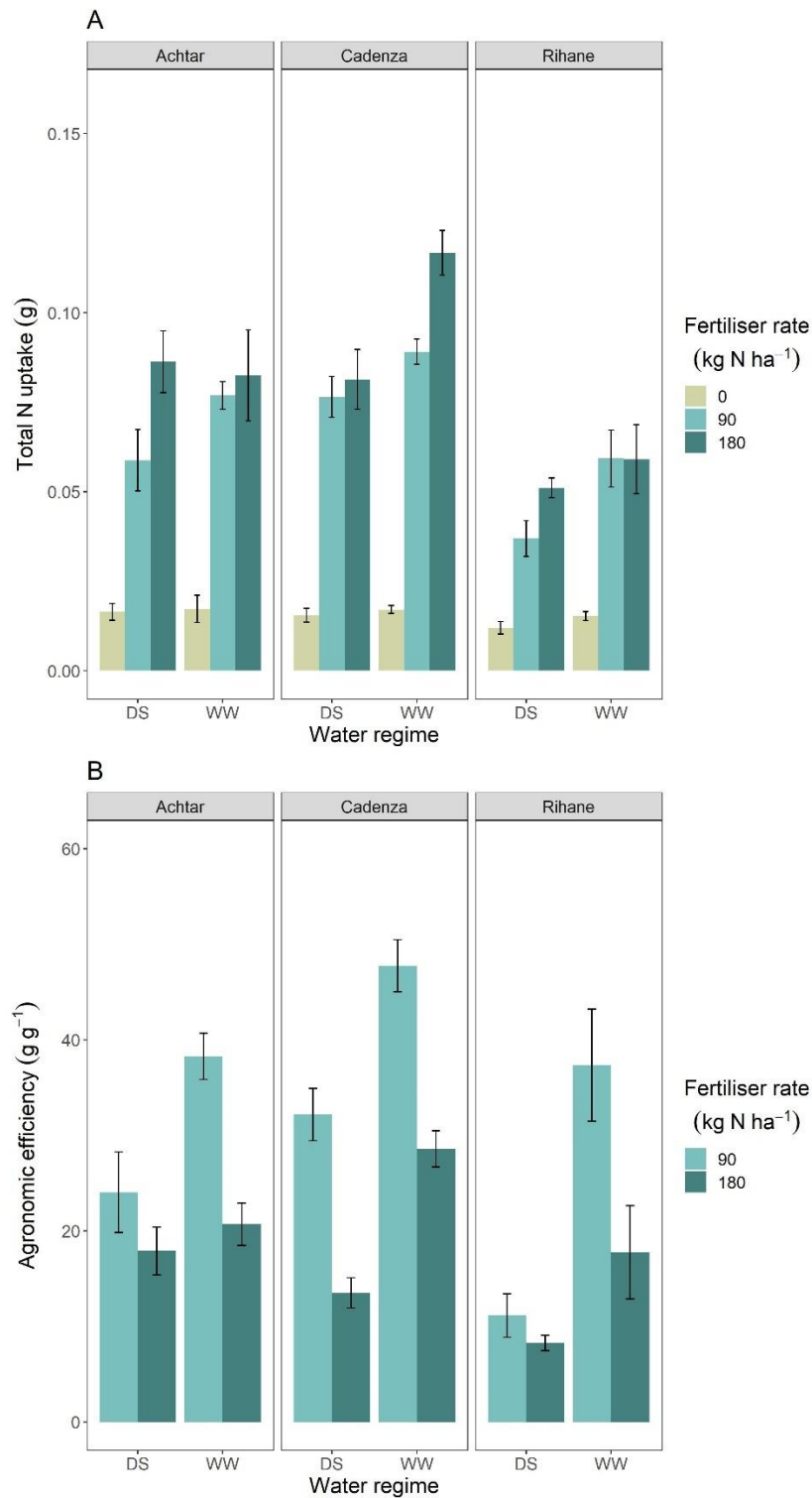


Figure 4-6: (a) Total plant N uptake by wheat variety under drought stressed and well-watered conditions at different N fertiliser rates, and (b) agronomic efficiency of N fertiliser by wheat variety under drought stressed and well-watered conditions at different N fertiliser rates. Data are means $\pm$ standard errors ($n = 5$). Letters depict significant difference between means ($p < 0.05$).

4.5 Discussion

4.5.1 Drought stress influence

Drought stress can affect plants at all stages of development, however, for cereal crops, panicle development and flowering stages are more sensitive to drought (Prasad et al., 2008). Several authors have reported decreased N metabolism enzymes and shoot biomass under drought stress, in wheat, sweet potato, and Chinese fir plantlets (Li et al., 2020; Xia et al., 2020; Ru et al., 2023). In the current study, wheat N assimilation in response to drought depended on variety and N fertiliser rate and type. Wang et al. (2017) found that drought impact on N assimilation in maize plant leaves was more pronounced on older and mature leaves, where photosynthesis is more limited, compared to younger leaves. In the current study, photosynthesis in the sampled flag leaves was probably not sufficiently restricted to reveal a significant effect of DS on some leaf N components. Additionally, plants develop different mechanisms to minimise oxidative damage including stress-defence-related proteins by upregulating protein production, which can increase with the duration of drought (Xu et al., 2021). In the current study, wheat leaf response to DS could explain the increased leaf protein concentration for unfertilised plants at the second sampling event, as they would be more stressed by drought as observed with leaf biomass reduction under DS at sampling event 2. Increased leaf protein after 16 days of water stress was also reported by Xu et al. (2021).

Furthermore, over the wheat growing period, the induced DS at tiller elongation generally restricted wheat development (tiller number) and productivity (dry biomass and grain weight) compared to plants that were WW, as has been reported by others (Prasad et al., 2008; Duma et al., 2022). This results in lower grain yield resulting from the negative effect on biomass production, as the mobilized N in leaves will be translocated for grain production and development (Xu et al., 2012). In the current study, grain weight was reduced by 46% under the DS treatment, which compares well with the recent meta-analysis of Wan et al., (2022), who reported that overall, drought stress reduced grain yield by up to 57%. The observed reductions in wheat growth and yield under DS probably resulted from reduced N uptake. Significantly reduced N uptake and NUE indices of wheat under DS has been previously reported for other crops (Hoseinlou et al.,

2013; Allende-Montalbán et al., 2021; Riache et al., 2022). Soil water availability affects the transport of N to absorbing roots by mass flow and diffusion of NH_4^+ and nitrate (NO_3^-) ions in the soil solution (Prasad et al., 2008). This is restricted under drought, with subsequent effect on plant growth, yield and NUE resulting in a greater residual soil N concentration. However, this may become available to subsequent crops as noted by Allende-Montalbán et al. (2021).

4.5.2 Variety effect

Nitrogen assimilation varied between varieties, and leaf N component concentrations tended to be greater in Cadenza and Rihane, relative to Achtar. Furthermore, Cadenza had the best yield potential and drought tolerance traits. Variety differences in grain yield under normal irrigation and deficit irrigation were reported by Beral et al. (2022), whereas Souza et al. (2004) only observed differences under normal irrigation. Plant adaptation to drought stress includes drought tolerance and drought recovery during growth following a drought period (Chen et al., 2016). In the current study, Cadenza was most drought tolerant, with a less negative effect of drought on growth and tiller development. Cadenza and Achtar had a drought recovery response with the development of additional tillers and heads following the drought, which was not evident in Rihane. Similar drought recovery was reported by Ramdani et al. (2010) in Moroccan conditions with emerging new tillers and spikes from a group of Moroccan wheat cultivars, including Achtar and Rihane. Achtar is particularly appreciated by farmers in Morocco for its adaption and performance under various stresses (Jlibene, 2006). Cadenza is a UK variety selected for its high-level N use trait (Hawkesford and Riche, 2020), so it is unsurprising that it showed the best overall RE_N of 67%. Rihane had the lowest RE_N but was still within the typical range of 30 - 50% (Dobermann, 2007; Rawal et al., 2022). The PE_N , and IE_N was similar across the three varieties, which were also within the typical ranges of 40 - 60 kg yield increase kg^{-1} increase in N uptake and 30 - 90 kg yield kg^{-1} N uptake, respectively (Dobermann, 2007).

4.5.3 Influence of nitrogen fertiliser type and rate

Fertiliser type

Similar to the findings of previous studies (Li et al., 2020; Ru et al., 2023), application of N fertiliser enhanced plant N assimilation under WW and DS. Compared to U, the application of US caused a significant accumulation of urea in leaf tissues, which increased with a greater N rate. This urea accumulation has been reported in peas, wheat, and maize and resulted from the inhibition effect of NBPT on plant leaf urease enzyme activity, potentially causing urea toxicity with concurrent effects on GS activity, NH_4^+ concentration, amino acids and protein profile in roots and shoots (Artola et al., 2011; Cruchaga et al., 2011; Zanin et al., 2016). During sampling event 1 and 2, GS activity explained about 36 and 40%, respectively, of the increase in dry biomass (Supplementary information Table D-4 and D-5) which was reduced under US treated plant. These results support earlier findings that NBPT can induce urea accumulation and impairments in GS activity and NH_4^+ assimilation (Artola et al., 2011; Cruchaga et al., 2011; Zanin et al., 2015).

Wheat growth and yield were also improved under both WW and DS conditions, which is in accordance with findings from field trials conducted in the Mediterranean region, including Morocco, Jordan, North-eastern Spain, and Southern Italy (Savin et al., 2022). In contrast to many previous studies (Sha et al., 2020; Folina et al., 2021) we did not observe increases in wheat growth and grain yield under US relative to U. Although, our findings were in agreement with Grant (2014), who reported no benefit of using urea treated with UI in the growth or yield of wheat over a three-year field experiment. Additionally, there was no significant difference between U and US on total N uptake, NUE indices and N balance, contrary to the findings of Wallace et al. (2020). This may have been because under our experimental conditions, both U and US provided sufficient N for growth and grain production. A meta-analysis by Rose et al. (2018) showed that enhanced efficiency fertilisers such as US have limited potential to enhance yield at recommended N rates for conventional N fertiliser. Lasisi et al. (2022) also reported statistically similar N uptake and NUE for Canola and wheat when they received fertilisers with and without a UI, which they related to N surplus coming from soil residual and mineralised N, hindering the advantage of using the UI.

Fertiliser rate

We observed greater leaf urea and NH_4^+ concentration at fertiliser rates of 180 kg N ha^{-1} relative to 90 kg N ha^{-1} , particularly under US. The high N applied at the stem elongation stage (140 kg N ha^{-1} in experiment 1) before leaf sampling could explain the high urea accumulation in the leaves of plants receiving 180 kg N ha^{-1} . Additionally, cytosolic GS activity catalyses NH_4^+ assimilation (Xia et al., 2020) derived from root N uptake or internal N recycling (Xu et al., 2012), and its downregulation by NBPT has been reported to slow down leaf NH_4^+ assimilation, leading to accumulation in the leaf (Cruchaga et al., 2013). Furthermore, N application at 180 compared with 90 kg N ha^{-1} increased wheat biomass production but not grain yield, although wheat N uptake increased with increasing N rate as observed in our study and elsewhere (Ierna et al., 2016; Hawkesford and Riche, 2020). Several authors have reported the lack of wheat yield response to increasing N rate (Raun et al., 2011; Hawkesford and Riche, 2020; Walsh et al., 2022). Thus, all determined NUE indices decreased as the applied N rate increased, as is commonly reported and in accordance with Tilman et al. (2002).

4.5.4 Combined effect of variety, water regime and N fertiliser treatments

In this study, Achtar leaf urea concentration was not influenced by application and rate of US fertiliser in sampling event 2, in contrast to Cadenza and Rihane revealing a potential rapid recovery of Achtar from NBPT impairments. This was unexpected, although a lack of effect of NBPT on spinach leaf N assimilation has been noted (Cruchaga et al., 2011). Further study on the influence of NBPT on N assimilation such as urease enzyme activity, amino acids profile in Achtar and other wheat varieties under various growth conditions is needed to elucidate the mode of action of NBPT and the reason for varietal differences. Nevertheless, at sampling event 2, there was a significant decrease in leaf urea concentration and an increase in GS activity for all varieties compared with values at sampling event 1. A temporary negative influence of NBPT on plant N metabolism, lasting about 10 days, has been reported by Krogmeier et al. (1989), Artola et al. (2011), and Haque et al. (2022), after which urease enzyme activity in shoots recovers; however, in roots, urease enzyme activity can take longer to recover from NBPT impairments (Cruchaga et al., 2011).

In Experiment 2 biomass and grain yield were greater under U than US at 180 kg N ha^{-1} , similar to Experiment 1, presumably due to the temporary inhibitory effect of NBPT

on N availability in the soil and assimilation in the leaf, reducing early biomass accumulation (Grant, 2014). The third urea dressing notably promoted Achar recovery following DS through tiller production. Allende-Montalbán et al. (2021) found that when N was split applied to maize, plants recovered faster from the temporary N deficiency after the second dressing with urea. Additionally, at 180 kg ha⁻¹ of applied U, there would be more N readily available and taken by plants to produce biomass. High N supply has been reported to alleviate drought stress in various crops (Agami et al., 2018; Iqbal et al., 2020; Wan et al., 2022), which was evident in the current study in the increased N uptake and grain production in Achar at 180 kg N ha⁻¹ under the DS treatment, suppressing the yield gap between DS and WW. The influence of US application on drought attenuation was only observed on tiller production, with no effect on Rihane, the most drought-sensitive variety in our experiment. Allende-Montalbán et al. (2021) reported an attenuation of the negative effect of drought stress on plant height when applying urea with a urease inhibitor whereas Gou et al. (2017) reported increased maize dry matter with the application of NBPT under DS condition.

Various factors can influence plant NUE indices, such as environmental conditions and management practices (Dobermann, 2007). Wallace et al. (2020) noted that AE_N of a wheat crop in Australia was mainly linked to PE_N which was influenced by variations in N management and cropping season. The AE_N in the current study showed greatest correlation with RE_N (86 %; Supplementary information Table D-6), as also reported by Wallace et al. (2022). According to Ferreira et al. (2022), increasing N availability in the soil by adding N fertiliser under water-stressed conditions improves N uptake, yield and NUE. This was only evident for Cadenza in the current study. The noticeable difference between Cadenza and the Moroccan varieties in N responsiveness might be linked to their selection criteria. Cadenza originated from the selection for N use efficiency (Hawkesford et al., 2014) whereas the Moroccan varieties have been selected mainly for their tolerance of various abiotic and biotic stresses and yield performance (Jlibene, 2006). Application of US did not influence N uptake, yield or NUE under DS, contrary to the study of Allende-Montalbán et al. (2021), who reported increased NUE with the use of a urease inhibitor under a water-stressed environment. Further studies, including much lower N application rates, would be useful to assess whether the US at a lower application rate could achieve the same yields as U at the recommended rate, thereby increasing NUE.

The findings of Drulis et al. (2022) support applying lower rates of N ($<180 \text{ kg ha}^{-1}$) with the use of UI for maize production.

Overall, there was no strong evidence that US fertiliser enhanced wheat tolerance to drought and improved NUE under the rates and conditions included in this study. Although wheat plants recovered from NBPT impairments on N metabolism, this study revealed possible biomass and grain yield reduction resulting from N assimilation impairment. This raises the importance of knowing the right time to supply wheat with US fertiliser for optimum plant N uptake and growth, considering the delayed release of N from soil and in plant. Farmers would benefit from the use of UI with urea fertiliser by adopting farm-specific fertiliser management, enabling timely application of fertiliser considering crop need during the growth cycle and weather conditions. Afshar et al. (2018) argued that timely application of UI and NI with urea considering meteorological conditions could enhance agronomic efficiency and reduce NH_3 volatilisation relative to untreated urea in dryland farming. Research on adequate N fertiliser management with associated water saving strategies to enhance crop tolerance to drought and productivity may be required in different regions with the projected increased frequency of drought. Although overall, Cadenza showed better growth, grain production, RE_N , and AE_N under the established growing conditions, and could be a variety of interest in drought-prone areas. Achtar had a good recovery potential after DS, an important drought coping mechanism. Residual soil N was greater under DS and at the highest N rate. Therefore, it is important to determine the optimum N rate for specific crops to minimize N losses and excess N application, particularly under dry and warm climatic conditions reported to cause important N losses from surface applied urea and where the efficacy of urease inhibitors to reduce NH_3 volatilisation is greatly reduced (Drame et al., 2023). Urease inhibitors are promising technologies, but they may need careful management to unlock their potential to boost crop yield and product quality.

4.6 Conclusion

Wheat production under water scarcity requires careful management of N fertiliser inputs. Drought stress had little impact on leaf N assimilation, but decreased plant biomass, grain yield and total N uptake, and therefore NUE, with marked differences between varieties. Application of N fertiliser enhanced N metabolism, growth, and grain production as well

as N uptake. Application of a urease inhibitor caused possible leaf urea toxicity and delayed N availability, limiting biomass accumulation and grain production, particularly at high N supply. However, plants recovered from the effects of the urease inhibitor, and total N uptake and NUE were not influenced by fertiliser type, but rather by the rate of applied N, wheat variety and water regime. We conclude that NUE in wheat under drought-stress can be improved by using N efficient varieties and timely applying a urease inhibitor at a low N rate. Furthermore, assessment of NBPT transfer to the food system such as cereals, horticultural products, and/or animal forages as well as the long-term effect on soil microbial community should receive more attention in future research.

4.7 Author contributions

Marieme Drame: conceptualization, methodology, investigation, data curation, formal analysis, visualization, writing – original draft, Writing – review & editing. **Maria Ozsvald:** conceptualization, methodology, investigation, Writing – review & editing, validation, supervision. **Alison Carswell:** conceptualization, methodology, visualization, Writing – review & editing, validation, supervision, Project administration, Funding acquisition. **William Roberts:** Writing – review & editing. **Jessica Hood:** Methodology, formal analysis, Writing – review & editing. **Martin Jemo:** Writing – review & editing, supervision, Funding acquisition. **Mark Pawlett:** Writing – review & editing, supervision, Funding acquisition. **Guy Kirk:** Writing – review & editing, validation, supervision, Funding acquisition, Project administration. **Tom Misselbrook:** Writing – review & editing, validation, supervision, Funding acquisition, Project administration.

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5 Discussion

Urease inhibitors (UI) have proved to be a promising strategy in reducing ammonia (NH_3) emissions from urea applications to agricultural land and improving nitrogen use efficiency (NUE), but little is known about their effectiveness under hotter, drier conditions, and potential interactions with plant nitrogen (N) metabolism, plant nitrous oxide (N_2O) emission and plant NUE under water scarcity. Therefore, the work of this thesis focussed on exploring the efficacy of UI in mitigating NH_3 and N_2O losses from urea fertiliser under warm and dry soil conditions (Chapter 2), particularly at temperatures reaching 35°C and soil moisture lesser than 40% water filled pore space (WFPS) have received less attention. Additionally, this work proposed to determine plant contribution to N_2O emissions and mainly inform on whether UI influence plant N_2O emission in the environment (Chapter 3). Furthermore, the influence of UI on plant NUE under water stressed condition and the potential to enhance plant tolerance to drought was assessed to identify potential further benefits of using UI (Chapter 4).

This section synthesises the main research findings and concludes by providing areas of research for future work.

5.1 Effect of soil processes on fertiliser nitrogen losses and uptake

5.1.1 Temperature and soil moisture on NH_3 and N_2O emissions

Urea hydrolysis in soil requires moisture availability either before or after application of urea granules on the soil surface. The activity of the urease enzyme catalysing this reaction is enhanced at high temperature like most microbial activity and respiration (Signor and Cerri, 2013). Therefore, the increase in soil temperature boosts the hydrolysis rate resulting in a rapid rise in pH around the urea granule as ammonium (NH_4^+) or NH_3 is formed. The ionic balance shifts from NH_4^+ to NH_3 as soil pH rises due to increased demand for protons during hydrolysis (Rochette et al., 2009; Sommer et al., 2004). Increasing temperature also favours water evaporation from soil pores enabling upward diffusion of NH_3 when urea is not incorporated deep into the soil yet. As a result, the increase in soil temperature (15°C up to 35°C) greatly increased NH_3 volatilisation in this study (Chapter 2), and the rate of volatilisation was also greater in soil with a lower soil moisture content for both urea and urea treated with UI. Additionally, the persistent high

temperature of 35°C over the 30 days of incubation induced continued NH₃ volatilisation for up to 25 days in contrast to that at lower temperatures. Lower NH₃ volatilisation at cooler temperatures and in soil with a greater moisture content is because of the lower rate of microbial activity coupled with a greater downward water movement displacing urea deeper in the soil (Rawluk et al., 2001).

Nitrous oxide emission, which occurs predominantly during microbial nitrification and denitrification processes, is also driven by the effects of soil temperature and moisture on the activity of nitrifying and denitrifying microorganisms (Signor and Cerri, 2013). Generally, N₂O emissions increase exponentially with rising soil temperatures (0 - 50°C) (Liu et al., 2011). In Chapter 2, greater emission of N₂O was measured at higher soil temperature (35°C). The soil moisture contents used in the study (20, 40 and 60% WFPS) would have favoured the nitrification process which requires aerobic conditions; under conditions of lower soil O₂ availability in saturated soil (>70% WFPS) N₂O emissions mainly derive from denitrification (Ruser and Schulz, 2015). From 15 to 25°C, soil moisture had a greater influence on cumulative N₂O emission than temperature; however, at the higher temperature (35°C) there was a soil moisture effect with greatest emissions at 60% WFPS. This demonstrates the increased soil microbial activity, probably for both nitrifying and denitrifying microorganisms, under higher soil temperature and moisture conditions (Signor and Cerri, 2013).

5.1.2 The efficacy of urease inhibitor in reducing NH₃ and N₂O emissions

Urease inhibitors inclusion with urea fertiliser is a promising technology with regard to reducing NH₃ volatilisation losses from urea fertilisers. Ammonia volatilisation was effectively reduced by the inclusion of UI with urea under all the examined soil temperature and moisture conditions in this study (Chapter 2). The half-life of a UI is an important factor determining the effectiveness in reducing nitrogen (N) losses. The increase in temperature from 15, 25 and up to 35°C reduced the efficacy of both studied UI. The UI assessed in this study (NBPT and NPPT) are degradable organophosphorus compounds (Byrne et al., 2020) and increasing soil temperature accelerates their degradation through increased soil microbial activity. A lack of sufficient soil moisture also reduced the effectiveness of the UI in minimising NH₃ volatilisation. Under low soil moisture conditions, such as 20 and 40% WFPS, urea hydrolysis would mainly occur in

the upper surface of the soil with less diffusion of the UI throughout the soil profile. Additionally, microorganisms are more abundant in the topsoil (Hao et al., 2021), therefore urea hydrolysis rate may surpass the UI inhibition rate. More insight into the influence of soil moisture on UI degradation and its half-life is needed, which has been better documented for temperature and soil pH. The hypothesis that UI would be more efficient in reducing NH_3 volatilisation under conditions favouring NH_3 volatilisation was not confirmed in this study. Nevertheless, UI effectively mitigated NH_3 volatilisation at high and low soil moistures, but the percent reduction was greater at lower temperature (15°C) and higher soil moisture (60% WFPS). This temperature and moisture condition would have reduced soil microbial activity and the UI degradation rate, whilst enabling downward diffusion of urea into the soil profile. Thus, the release of NH_4^+ would have happened deeper in the soil, slowing the upward diffusion of NH_3 (Grant et al., 1996). Although not specifically relevant to this study, the pH (6.2) of the soil used in the study (Chapter 2) was slightly acid which could have also contributed to the degradation the UI in soil because NBPT is reported to principally degrade via chemical hydrolysis under acidic conditions (Byrne et al., 2020).

Urease inhibitors are intended to reduce N losses through a reduction in NH_3 volatilisation. Nevertheless, by delaying the release of NH_4^+ into soil solution, nitrification and denitrification rates would also be limited, hence UI can also reduce N_2O emissions (Sanz-Cobena et al., 2014). In this study, N_2O emission was not affected by the application of UI as reported in the meta-analysis of Akiyama et al. (2010). Under such circumstances, application of double inhibitors such as UI and nitrification inhibitors (NI) could be an option for reducing NH_3 and N_2O simultaneously. However, the reduction of NH_3 losses may be less pronounced than when UI is used alone (Soares et al., 2012) as NI would maintain NH_4^+ longer the in soil through the delay of nitrification. The compensation in such a scenario would be a reduction in N_2O emission by the NI.

5.1.3 Effect of soil condition on plant nitrogen use efficiency

Nitrogen, present or added to the soil, undergoes various transformations which dictate its availability to plants. Nitrogen available in soil for plants in the form of NH_4^+ and nitrate (NO_3^-) are water soluble and water supply influences their potential movement in the soil profile (Davidson, 2018). Providing sufficient water after N surface application

enables movement of inorganic N to the plant root zone through diffusion or mass flow (Farzadfar et al., 2021). In addition to being taken up by the plant, some of the N may be fixed by soil particles, precipitated out of solution, or leached (Davidson, 2018). The applied drought stress in Chapter 4 reduced plant N uptake, growth, and productivity and nutrient mobility to the surface of plant roots was clearly affected. The findings from Chapter 2 support this fact given that at the lower soil moisture contents of 20 and 40% WFPS, there was generally greater NH_3 volatilisation (due to urea hydrolysis on the soil surface) but also there was a higher N concentration remaining on the soil surface (0-2.5 cm depth) after 30 days of incubation. Furthermore, the nutrient balance determined in Chapter 4 showed that there was a significantly greater amount of N left in the soil under the drought-stressed (DS) compared to well-watered (WW) treatment. Findings from Chapters 2 and 4 demonstrate that water scarcity can cause important N losses through NH_3 volatilisation but also undermine plant N acquisition, growth, and productivity. Therefore, N fertiliser management under water limited conditions is critical and could justify the use of enhanced efficiency fertilisers (EEF) such as UI which have the potential to reduce N losses and prolong soil N availability under different soil moistures.

Although the UI assessed did not improve plant N uptake, NUE or tolerance to drought under the experimental conditions of Chapter 4, several other studies have observed enhanced N uptake, NUE and increased yield (Abalos et al., 2012). The hypothesis of the study presented in Chapter 4 was based on earlier studies (Gou et al., 2017; Haque et al., 2022) which have reported enhanced plant tolerance to drought when given foliar application of urea with NBPT; however, foliar application may not be applicable to all crops or growing conditions. Therefore, it makes sense to assess the potential of UI to enhance plant tolerance to drought with surface broadcast N fertiliser application for different crops. Studies to date suggest that UI use would be more beneficial at low N application rates, below conventionally recommended rates for N fertilisers (Rose et al., 2018). Applying N at a lower rate (90 kg ha^{-1}) enhanced NUE in Chapter 4. Therefore, further reducing the applied N rate may enhance the effectiveness of UI and their potential to improve drought tolerance through soil application.

Of the wheat varieties studied in Chapter 4, Cadenza might be a promising choice for wheat production in regions experiencing drought stress, due to its better growth and N

uptake potential as observed under the applied DS. Similarly, Achtar showed potential for recovery from DS condition. However, to validate these varieties in future variety selection programmes for drought tolerance, wider field studies are required.

5.2 Plant response to nitrogen fertilisers

5.2.1 Fertilisers influence on nitrogen assimilation and use efficiency

Application of N fertilisers enhanced N assimilation, growth, and yield in all wheat varieties under WW or DS conditions (Chapter 4). Plants can take up available inorganic (NH_4^+ , NO_3^-) and organic N such as amino acids and ureides (Farzadfar et al., 2021). Thus, when supplied with urea fertiliser and urea treated with UI, NH_4^+ and urea concentrations and glutamine synthetase (GS) activity in plant leaves increased. Although, plants can assimilate these N forms, excess accumulation in the leaf can cause NH_4^+ or urea toxicity in plants. Several factors can alter plant N assimilation as discussed in Chapter 1, including the rate and type of applied N. Increased leaf urea concentration was observed at the high urea application rate and when NBPT was added with the applied urea (Chapter 4). The potential inhibitory effect of NBPT on soil urease and plant internal urease activity was confirmed in Chapters 2 and 4, respectively. Therefore, NBPT inhibits both soil and plant urease activity, with a subsequent delay in the release of soil NH_4^+ for plant uptake and restricted leaf urea assimilation. Although, the inhibition effect was temporary in soil as well as in the plant, the simultaneous inhibition effect may have contributed to the observed reduction in wheat biomass and grain yield, particularly at the highest rate of applied N (180 kg ha^{-1}) because plant N uptake, and probably NBPT absorption, was greater at this rate. Therefore, applying a high rate of urea treated with NBPT may not be beneficial in terms of crop productivity. Plant response to N fertilisers varied between wheat varieties (Chapter 4), and both Cadenza and Rihane had higher leaf urea concentration at the highest N application rate and when NBPT was applied. In Rihane, the accumulation of leaf urea in the presence of NBPT also reduced leaf GS activity that led to an accumulation of leaf NH_4^+ , particularly at the high N rate. The variety Achtar differed in that there was no effect of NBPT on leaf urea or NH_4^+ concentrations. These findings reveal that application of NBPT may not only influence plant urease activity, but also GS activity depending on the wheat variety. The influence of fertiliser treatments (type and rate) on N assimilation was more evident under WW

growth conditions, with no significant effect on leaf N components under DS conditions in this study, which may be due to compensation mechanisms (N recycling) that plants develop under stress (Xu et al., 2012).

5.2.2 Fertilisers effect on plant N₂O emission

In cropping systems, N₂O emission is generally estimated from the soil-plant interface. Plants can contribute to soil N₂O emission by modifying soil characteristics (Gao et al., 2019) and through N uptake (Chen et al., 2021). Lower N₂O emissions have been observed in fields cultivated with varieties with high N use efficiency (Chen et al., 2021). Efficient uptake of NH₄⁺ and NO₃⁻ by plants can reduce the soil concentration of N available for nitrification and denitrification, therefore resulting in less N₂O emission. Plants have been reported to produce N₂O during NO₃⁻ assimilation in the leaf chloroplast (Smart and Bloom, 2001). Thus, the form of N taken up by plants influences the release rate of N₂O in plants (Lenhart et al., 2019). In this study (Chapter 3) and in several other studies, plants fertilised with NH₄⁺ did not emit as much N₂O as plants supplied with NO₃⁻. The findings from the hydroponic experiment (Chapter 3) did not provide evidence that N₂O was produced in plant leaves as the applied nitrate inhibitor did not alter N₂O emission. However, the soil experiment showed that the presence of shoots (leaves) augmented N₂O emissions.

Moreover, N₂O emission in the soil experiment (Chapter 3) was lower in Cadenza plants compared to Sokoll, with GS activity being higher in Cadenza. Higher GS activity signifies a higher rate of NH₄⁺ assimilation into protein and other metabolites in the leaf (Zhang et al., 2017). Therefore, during growth leaf NH₄⁺ concentration was much higher in Sokoll than in Cadenza. Additionally, the fact that under low N growth conditions Cadenza developed a greater shoot biomass compared to Sokoll (Chapter 3) suggests an improved NUE in comparison to the assessed Moroccan varieties (Chapter 4). Cadenza was the variety used in the hydroponic study (Chapter 3) and this observed trait of Cadenza to maintain efficient N use under different stress conditions (drought, NBPT, low N) may have hidden its response to the applied nitrate reductase (NR) activity inhibitor, although, the plants receiving GS activity inhibitor had reduced leaf GS activity. Sokoll emitted more N₂O under low N growth conditions, but there is no robust information in this study to help explain this finding. Nonetheless, based on previous

studies, it could be related to the plant response to low N supply and its influence on the various plant N metabolism processes (Lenhart et al., 2019). The ability of plants to assimilate N may have a direct or indirect relationship with N₂O emission from the soil-plant interface. Further investigations are required to fully understand the relationship between plant NH₄⁺ and NO₃⁻ assimilation and N₂O emission, and to determine the influence of growth conditions on these processes.

The study in Chapter 3 was probably limited by the N₂O measurement methods (chamber system) and the inhibitor application methods (in solution for root uptake rather than foliar application). The observed N₂O emission rates were low and close to the detection limit of the Picarro G2508 gas analyser (Picarro Inc., Santa Clara, CA, United States) (range 0.3-200 ppm). Therefore, using a more sensitive detection method for trace gas emissions from plants would probably reduce data variability.

5.3 Contribution to knowledge and impact

This study has shown that the efficacy of UI (NBPT and NPPT) is greatly reduced at high temperatures and low soil moisture (Chapter 2). Therefore, to improve NUE under these conditions, additional climate smart practices and technologies could be combined with the use of urea and UI. For crop production in warm and dry areas, using urea fertiliser treated with UI inhibitors without accompanied fertiliser management practices may reduce the economic benefits they could obtain from the use of UI. Determining the economic benefits of using UI in these areas may be relevant. Furthermore, adapted storage conditions of fertilisers treated with UI may be needed in warm regions as it has been documented that temperature can reduce UI longevity during storage period.

The study has also added to our knowledge of direct plant contribution to N₂O emissions as a result of plant N assimilation processes in relation to wheat varieties, and that UI does not influence plant N₂O emission. However, the rate of N₂O emission may be greater from N fertiliser additions under low N initial growth conditions (Chapter 3). The translation of these knowledge would be that using plant varieties that have high N assimilation rate such as Cadenza could reduce N₂O losses, particularly, when potassium nitrate is to be applied to plants grown in soil with relatively low N.

From Chapter 4, it is evident that the application of UI to wheat may not enhance tolerance to drought or increase wheat N uptake and NUE. Additionally, this work showed that the effect of NBPT on leaf N assimilation in wheat is variety dependant. The application of NBPT to wheat can influence both leaf urease and GS activity, particularly at a high N application rate. Additionally, wheat can recover from the NBPT effect on urea metabolism over time, but the inhibitory effect on both soil and plant processes can result in a yield reduction. Furthermore, drought stress caused higher soil residual N. For producer perspective, using N efficient varieties such as Cadenza could both increase yield and reduce N losses to the environment. This applies particularly under drought stressed condition, where varieties that have both or either N use efficiency or drought tolerance would optimise productivity. Urease inhibitors are promising technologies, but they may need careful management to unlock their potential to boost crop yield and product quality.

5.4 Future work

The work conducted in this thesis was laboratory based. A logical next step would be to upscale the research in the field as the observed effects may depend on other soil characteristics (pH, cation exchange capacity, organic matter, soil type, etc.), meteorological conditions, environmental and management factors.

The findings in this study suggest treatment of urea with UI is a promising mitigation strategy for reducing NH_3 in warmer and drier climates. However, due to the rapid degradation of UI under high temperatures its efficacy may be limited, therefore exploring whether higher concentrations of NBPT may be effective under warmer and drier conditions could be worthwhile. However, because of the possible transfer of NBPT into the plant, the dosage should be within environmentally safe recommendations, depending on the cropping system (e.g., cereal cropping, grassland). Assessing NBPT transfer to cereal products and/or animal forages as well as the long-term effect on soil microbial community should therefore be included. Moreover, novel fertiliser technologies (including chemical and biological) that have a longer life span under the more extreme climatic conditions being experienced worldwide could be explored.

Climate change scenarios predict more frequent droughts in many regions (IPCC, 2023) with probable important impact on nutrient availability in soil and plant growth.

Using crop varieties with the potential to adapt to drought stress through various mechanisms (tolerance, escape, recovery) and with the ability to efficiently use available and supplied nutrients could maintain productivity. Reynolds and Langridge (2016) emphasised the complexity of designing new crop varieties because of the unpredictable interactions of genotype–environment–crop management ($G \times E \times M$) largely driven by future weather (E), farmer choices (M), and specific location ($E \times M$). Thus, field assessment of wheat varieties (and other subsistence crops) adapted to drought and with efficient N use is essential. The review of Foulkes et al. (2009) revealed that the promising traits for selection to increase NUE may be different for both feed and bread-making wheat varieties. Therefore, further selection for NUE and drought tolerance is essential. More importantly, site specific (and variety specific) management of N should be encouraged such as the adoption of computer-based decision support tool (e.g., Nutrient Expert software) for nutrient management (Pampolino et al., 2012). Best management practices such as the 4R management strategy should be encouraged because of the higher residual N under drought conditions and due to the potential delay in N availability for plant uptake when urea is treated with UI. For instance, tailoring nitrogen rates according to soil fertility tests ensures that the crop receives adequate nitrogen without over-application. For EEF, single or split application of N should be considered depending on crop need throughout the growth cycle and on the nutrient release rate to meet crop demands. Under low soil moisture conditions, applying fertiliser through localised application (e.g., band application) or where fertigation is available, can improve nitrogen use efficiency (Bhattarai et al., 2008). Water conservation techniques such as micro- or drip-irrigation, subsurface irrigation and zai pits (i.e., planting pits around the plants generated to capture rain or irrigated water) can be used as an effective and efficient way to conserve moisture and enhance water use efficiency in dryland agriculture (Rahman, 2017). Conservation farming systems based on non-inversion tillage methods can be adopted in drought-prone areas as water harvesting techniques to enhance water retention as demonstrated by Rockström et al. (2009) in semi-arid and dry sub-humid locations in East and Southern Africa. Additionally, more attention should be given to the variety Achtar to understand the rapid leaf recovery from the accumulated urea in the presence of NBPT.

Nitrous oxide emission from agricultural lands and other anthropogenic activities has greatly contributed to global warming (Tian et al., 2020). To mitigate N₂O emission through emission reduction, it is essential to identify and understand the processes of N₂O production from different sources (Timilsina et al., 2020). Plants contribute to N₂O emissions, and the N assimilation processes, including NR and GS activity, could be the main driver. Therefore, further studies should be designed to explore plant N metabolism in relation to N₂O formation in the leaf. New approaches, such as metabolomics and computational approaches could provide a better understanding of N metabolism (Beatty et al., 2016) and, potentially, plant N₂O emission.

5.5 References

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Appendices

Appendix A Ethical approval letter



13 April 2024

Dear Miss Drame ,

Reference: CURES/21627/2024

Project ID: 24872

Title: Impacts of Urease Inhibitors on Nitrogen Assimilation in Wheat and on Reducing Nitrogen Losses

Thank you for your application to the Cranfield University Research Ethics System (CURES).

We are pleased to inform you your CURES application, reference CURES/21627/2024 has been reviewed. You may now proceed with the research activities you have sought approval for.

If you have any queries, please contact CURES Support.

We wish you every success with your project.

Regards,

CURES Team

Appendix B Supplementary Materials for Chapter 2

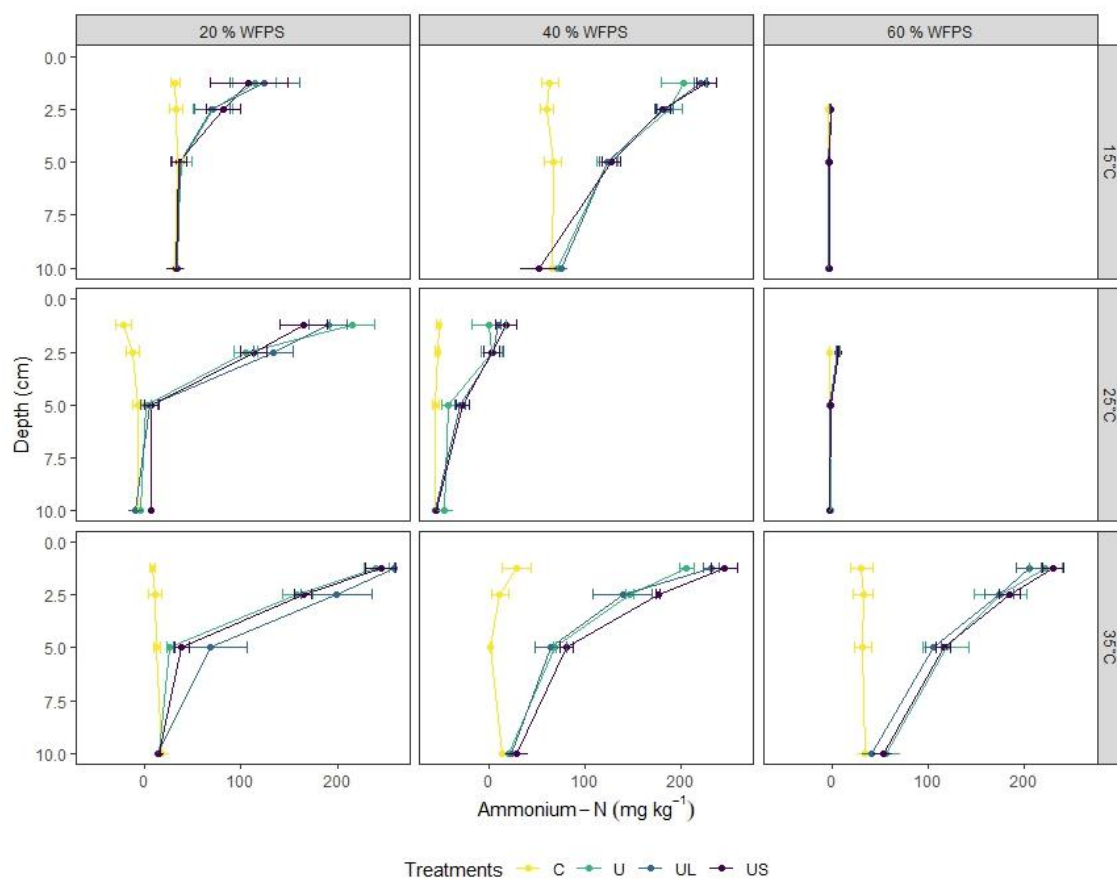


Figure B-1: Soil ammonium-N following incubation for the control (C), urea (U) and urea with urease inhibitor (UL and US) treatments at 15, 25, 35°C and 20, 40 and 60% water filled pore space (WFPS). Datapoints represent mean values and vertical bars depict standard error of the mean (n = 3).

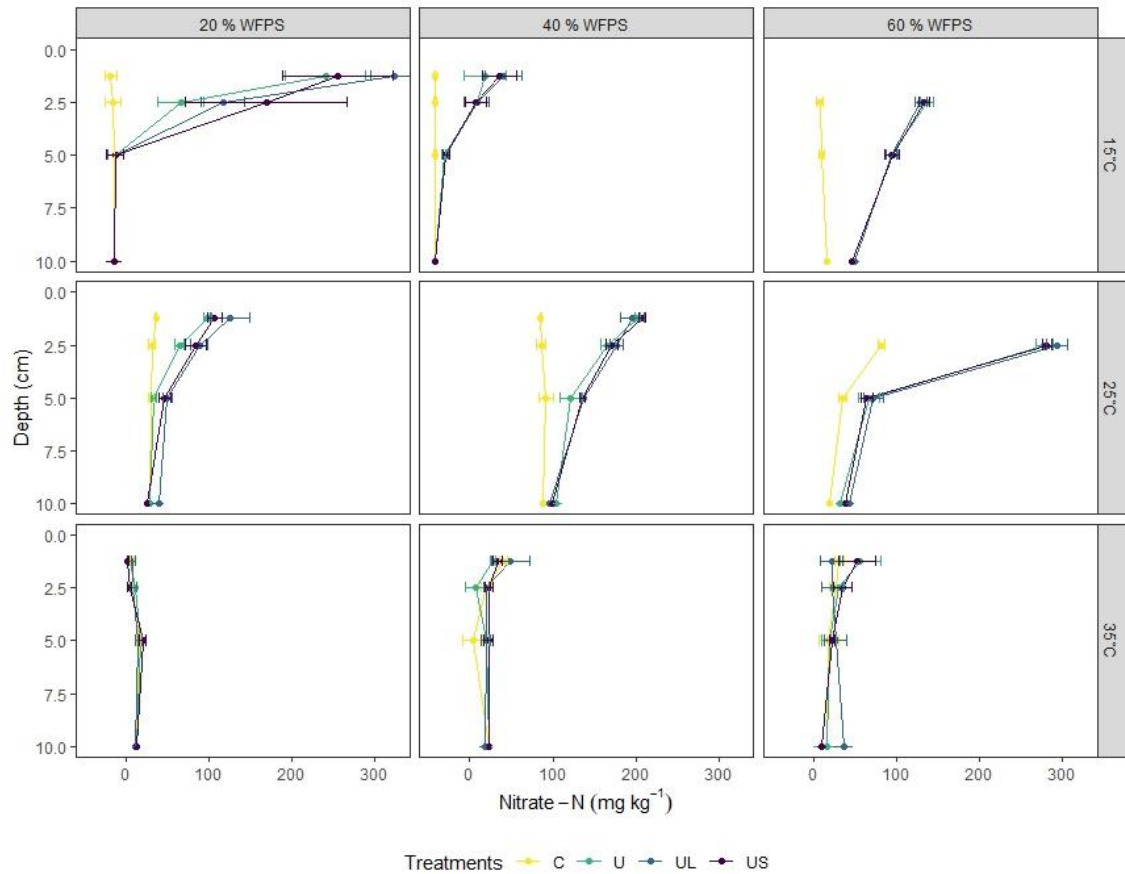


Figure B-2: Soil nitrate + nitrite-N at the end of soil incubations treated with 0-nitrogen control (C), urea (U) and urea with urease inhibitors (UL and US) at 15, 25, 35°C and 20, 40 and 60% water filled pore space (WFPS). Datapoints represent mean values and vertical bars depict standard error of the mean (n = 3).

Appendix C Supplementary Materials for Chapter 3

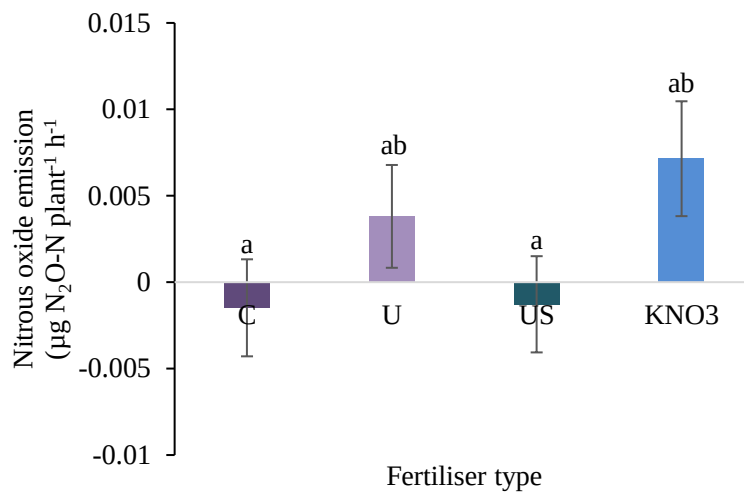


Figure C-1: Nitrous oxide emission in the experiment in soil from Roots+shoots as influenced by fertiliser. Bars are means $\pm$ standard error ($n = 6$). Letters depict significant difference between means ($p < 0.05$). The control treatment in (c) refers to Roots+Soil emission with no additional N.

Appendix D Supplementary Materials for Chapter 4

Table D-1: Significance of interactions according to linear mixed effects modelling between variety, water regime, and nitrogen source on wheat leaf N components at the first and second sampling events.

	NH ₄ S1 (μmol g ⁻¹)	NH ₄ S2 (μmol g ⁻¹)	Urea S1 (μmol g ⁻¹)	Urea S2 (μmol g ⁻¹)	GS S1 (μmol mg ⁻¹ h ⁻¹)	GS S2 (μmol mg ⁻¹ h ⁻¹)	Protein S1 (mg g ⁻¹)	Protein S2 (mg g ⁻¹)	Dry biomass S1 (g)	Dry biomass S2 (g)
<i>Significance of Interaction effects</i>										
Variety x Water regime	0.084	0.207	0.465	0.522	0.801	0.447	0.457	0.885	0.526	0.151
Variety x Fertilisation	0.224	0.902	0.190	0.668	0.440	0.061	0.976	0.144	0.750	0.452
Water regime x Fertilisation	0.847	0.731	0.290	0.462	0.807	0.525	0.609	0.030	0.622	0.079
Fertilisation x Fertiliser type	0.237	0.604	<0.001	<0.001	0.781	0.199	0.084	0.836	0.002	0.756
Fertilisation x Fertiliser rate	0.187	0.898	<0.001	<0.001	0.467	0.130	0.266	0.833	0.474	0.112
Variety x Water regime x Fertilisation	0.526	0.764	0.770	0.465	0.703	0.212	0.860	0.532	0.735	0.731
Variety x Fertilisation x Fertiliser type	0.518	0.537	0.083	<0.001	0.287	0.348	0.317	0.490	0.789	0.267
Water regime x Fertilisation x Fertiliser type	0.400	0.679	0.844	0.212	0.685	0.458	0.942	0.897	0.508	0.927
Variety x Fertilisation x Fertiliser rate	0.313	0.409	0.434	0.004	0.986	0.667	0.610	0.863	0.425	0.440
Water regime x Fertilisation x Fertiliser rate	0.851	0.309	0.665	0.818	0.052	0.693	0.293	0.724	0.975	0.909
Fertilisation x Fertiliser type x Fertiliser rate	0.008	0.563	<0.001	0.003	0.069	0.170	0.143	0.783	0.601	0.206
Variety x Water regime x Fertilisation x Fertiliser type	0.099	0.012	0.311	0.089	0.040	0.834	0.076	0.136	0.057	0.745
Variety x Water regime x Fertilisation x Fertiliser rate	0.164	0.873	0.988	0.300	0.996	0.664	0.326	0.760	0.957	0.614
Variety x Fertilisation x Fertiliser type x Fertiliser rate	0.518	0.087	0.189	0.182	0.935	0.238	0.769	0.250	0.051	0.901
Water regime x Fertilisation x Fertiliser type x Fertiliser rate	0.100	0.373	0.597	0.487	0.328	0.146	0.385	0.481	0.143	0.283
Variety x Water regime x Fertilisation x Fertiliser type x Fertiliser rate	0.548	0.003	0.247	0.318	0.979	0.512	0.771	0.331	0.864	0.177

S1 and S2 refer to the 1st and 2nd plant leaf sampling. Values are *p* values from REML analysis. The significance values were determined at the *p* < 0.05 level

Table D-2: Significance of interactions according to linear mixed effects modelling between variety, water regime, and nitrogen source on plant growth parameters, yield and N uptake.

	Tiller number during drought	Tiller number at harvest	Plant biomass (g)	Grain weight (g)	Total N uptake (g)
<i>Significance of Interaction effects</i>					
Variety x Water regime	0.028	<0.001	0.086	0.027	0.230
Variety x Fertilisation	0.078	0.037	<0.001	0.046	0.002
Water regime x Fertilisation	0.019	0.653	0.267	<0.001	0.110
Fertilisation x Fertiliser type	0.346	0.543	0.037	0.169	0.088
Fertilisation x Fertiliser rate	0.426	0.133	0.042	0.966	<0.001
Variety x Water regime x Fertilisation	0.570	0.264	0.502	0.405	0.716
Variety x Fertilisation x Fertiliser type	0.211	0.012	0.198	0.250	0.228
Water regime x Fertilisation x Fertiliser type	0.578	0.914	0.347	0.239	0.341
Variety x Fertilisation x Fertiliser rate	0.092	0.632	0.515	0.437	0.447
Water regime x Fertilisation x Fertiliser rate	0.606	0.877	0.623	0.235	0.659
Fertilisation x Fertiliser type x Fertiliser rate	0.082	0.550	0.403	0.026	0.081
Variety x Water regime x Fertilisation x Fertiliser type	0.222	0.010	0.773	0.479	0.870
Variety x Water regime x Fertilisation x Fertiliser rate	0.359	0.690	0.167	0.012	0.048
Variety x Fertilisation x Fertiliser type x Fertiliser rate	0.057	0.657	0.687	0.754	0.471
Water regime x Fertilisation x Fertiliser type x Fertiliser rate	0.535	0.072	0.122	0.481	0.261
Variety x Water regime x Fertilisation x Fertiliser type x Fertiliser rate	0.066	0.247	0.997	0.696	0.896

Values are *p* values from GMML and REML analysis. The significance values were determined at the $p < 0.05$ level.

Table D-3: Significance of interactions according to linear mixed effects modelling between variety, water regime, and nitrogen source on nitrogen use efficiency indices and N balance.

	Apparent recovery efficiency (%)	Physiological efficiency (g g ⁻¹)	Agronomic efficiency (g g ⁻¹)	Internal utilisation efficiency (g g ⁻¹)	Nitrogen balance
<i>Significance of Interaction effects</i>					
Variety x Water regime	0.211	0.599	0.046	0.274	0.609
Variety x Fertiliser type	0.433	0.305	0.580	0.441	0.847
Water regime x Fertiliser type	0.502	0.984	0.911	0.917	0.296
Variety x Fertiliser rate	0.239	0.621	0.373	0.828	0.984
Water regime x Fertiliser rate	0.318	0.337	0.359	0.235	0.614
Fertiliser type x Fertiliser rate	0.110	0.993	0.349	0.829	0.866
Variety x Water regime x Fertiliser type	0.803	0.789	0.648	0.799	0.522
Variety x Water regime x Fertiliser rate	0.202	0.533	0.043	0.593	0.409
Variety x Fertiliser type x Fertiliser rate	0.533	0.714	0.715	0.851	0.623
Water regime x Fertiliser type x Fertiliser rate	0.932	0.649	0.939	0.633	0.325
Variety x Water regime x Fertiliser type x Fertiliser rate	0.982	0.351	0.599	0.382	0.932

Values are *p* values from REML analysis. The significance values were determined at the *p* < 0.05 level.

Table D-4: Pearson correlation table of measured leaf components at sampling event 1

Dry biomass	-				
GS activity	0.3564	-			
NH ₄	0.162	0.0694	-		
Protein	0.1695	0.2162	0.0676	-	
Urea	0.1276	0.2022	0.3041	0.2189	-
	Dry biomass	GS activity	NH ₄	Protein	Urea

Table D-5: Pearson correlation table of measured leaf components at sampling event 2

Dry biomass	-				
GS activity	0.3986	-			
NH ₄	-0.1111	0.0765	-		
Protein	0.0184	0.3617	0.0049	-	
Urea	0.0243	0.1137	0.2722	0.0177	-
	Dry biomass	GS activity	NH ₄	Protein	Urea

Table D-6: Pearson correlation table of NUE indices

AEN	-			
IEN	0.5544	-		
PEN	0.5897	0.731	-	
REN	0.8616	0.2516	0.1508	-
	AEN	IEN	PEN	REN

Appendix E Ammonia Gas Sampling Protocol (used in chapter 2)

Measurements of ammonia emissions from soils using static throughflow chambers within the laboratory are made by capturing ammonia gas in acid traps and measuring the ammonium-N concentration of the acid trap. The accumulation of ammonia over a known period of time within the acid trap allows cumulative ammonia emissions to be measured.

E.1 Chamber

Equipment and checks:

- Chambers – check there are no cracks and that the seals on all chamber ports and around any join are in good condition.
- Chamber lids – check that gas sampling tube has been removed and the sampling port is plugged.
- Tubing – ensure all tubing is in good condition, without any kinks or squashed sections, and that the tubing is dry.
- Pump and flow meters – Before starting the pump, close all valves (turn clockwise until valve closes, do not over-tighten). Switch on the pump, then open each flow meter valve individually and fully to check that flow is evenly distributed between the 12 units, also listen for any air leaks (a hissing sound). Close each flow meter valve and turn the pump off.
- Glassware – check all flasks (150 mL Erlenmeyer flasks with Quickfit ground glass sockets) are clean and in good condition and that the Quickfit openings are intact. Check that all glass tubing is clean and in good condition, the glass tubing is fragile so take care when handling it.

E.2 Sampling

Equipment and preparation:

- Labelled sample vials for submitting samples to lab.

- 48 x 150 mL Erlenmeyer flask containing 80 mL of 0.02 M phosphoric acid (H_3PO_4) solution.
- 24 x Rubber bungs fitted with glass tubes (each bung contains a 150 mm x 6 mm and an 80 x 6 mm length x outer diameter glass tube).
- Recording sheet
- Watch, for recording start and end times.

Chemical and preparation:

Phosphoric acid (H_3PO_4) at 85 wt. % in H_2O extra pure. If a different concentration of H_3PO_4 is needed then the weight described below for making a 0.02 M H_3PO_4 solution will need to be adjusted accordingly, based on the density and weight percentage of the H_3PO_4 . Phosphoric acid (CAS: 7664-38-2) has a molecular weight of 98.00 g/mol and with a concentration of ≥ 85 wt. % in H_2O has a density of 1.685 g/mL at 25°C.

Calculations for 0.02 M H_3PO_4 preparation:

- Density of 1 L of 85 wt. % H_3PO_4 = 1685 g
- Grams of H_3PO_4 in 1 L of 85 wt. % H_3PO_4 : $1685 \times 0.85 = 1432$
- Molarity of H_3PO_4 at 85 wt. %: $1432/98 = 14.615$
- For 1 L of 0.02 M solution from 14.615 M H_3PO_4 solution: $0.02 \times 14.615 = 0.001368$ L, or 1.37 mL or 2.31 g ($1.368 \text{ mL} \times 1.685 \text{ g/mL}$)
- Half fill a 5 L volumetric flask with deionised (DI) water. Using a balance within a fume cupboard (with an acid filter, such as in the Air Lab; make sure a weight check has been performed that day prior to using the balance), weigh 11.55 g of 85% H_3PO_4 into a clean beaker, note the acid weight. Still working within the fume cupboard slowly pour the H_3PO_4 from the beaker into the 5 L volumetric flask. Not all H_3PO_4 will be transferred in this way. Therefore, wash the beaker with deionised water and transfer the washings into the 5 L volumetric flask, do this at least three times to ensure all acid is transferred to the volumetric flask. If the solution is warm allow to cool before making up to the 5 L volume mark with DI water.

- If any small spillages of the concentrated H_3PO_4 occur within the fume cupboard, dilute the spillage well with water before attempting to clean up with paper towels. Rinse any paper towels well under running water before putting in the waste.

Personal protective equipment:

- Safety goggles, nitrile gloves, lab coat with long sleeves and a fume cupboard with an acid filter are the required personal protective equipment for making this solution.

E.2.1 Sampling strategy

For a 12-chamber experimental system you will require 24 flasks containing 80 mL of 0.02 M H_3PO_4 solution (12 x inlet flasks and 12 x outlet flasks), in addition you will require a further 24 flasks of 0.02 M H_3PO_4 solution for the next change of acid traps. Frequency of acid trap changes will be dependent on the treatments applied, for example an organic N source such as cattle slurry will require more frequent changes immediately following application relative to an inorganic N source like urea fertiliser. Typically, with chemical N source acid traps are changed daily for weeks 1 and 2 and can then be changed every 2 days for weeks 3 and 4, following the amendment.

E.2.2 Starting an ammonia sample run

The samples consist of the inlet and outlet acid trap for each chamber. The inlet acid trap cleans the air entering the chamber, whereas the outlet acid trap collects any NH_3 gas which is produced from the soil. The period of time that an acid trap is used to collect NH_3 is used to determine cumulative NH_3 emissions and can be used to determine NH_3 fluxes.

- 2.2.1 Fit the rubber bungs with glass tubes to the 24 flasks containing the acid traps. Label all flasks with both the chamber number (1-12) they will be attached to and whether it is an inlet (in) or outlet (out) flask.
- 2.2.2 After applying treatments to the soil surface within chamber 1 cover the chamber with the associated throughflow lid (also marked as 1) and seal it with electrical tape to ensure an air-tight seal. Attach the inlet tubing to the inlet acid trap at the short glass tube (not the tube immersed in solution). Attach the outlet tubing to the

outlet acid trap at the long tube, immersed in solution. Then attach the short tube of the outlet acid trap to the tubing leading to the flow meters. *It is essential to double check these connections are to the right acid trap tubes otherwise the H_3PO_4 solution will be pulled through the system, and the system will require drying overnight before it can be used again.*

2.2.3 Ensure all flow meter valves are closed, switch on the vacuum pump. Once a vacuum has been achieved the flow meter valve associated with chamber 1 can then be opened slowly to achieve a flow rate of 1 L/min. Check that both the inlet and outlet acid traps are bubbling and record the start time of NH_3 sampling for chamber 1.

2.2.4 Apply treatment to the soil surface of chamber 2. Cover chamber with associated throughflow lid (marked as 2) and seal with electrical tape to ensure an air-tight seal. Attach the inlet tubing to the inlet acid trap at the short glass tube (not the tube immersed in solution). Attach the outlet tubing to the outlet acid trap at the long tube, immersed in solution. Then attach the short tube of the outlet acid trap to the tubing leading to the flow meters. *It is essential to double check these connections are to the right acid trap tubes otherwise the H_3PO_4 solution will be pulled through the system, and the system will require drying overnight before it can be used again.*

2.2.5 Slowly open the flow meter valve associated with chamber 2, to achieve a flow rate of 1 L/min. *You might have to adjust the other flow meter valves that are running to maintain all flow rates at 1 L/min.* Check that both the inlet and outlet acid traps are bubbling and record the start time of NH_3 sampling for chamber 2.

2.2.6 Repeat 2.2.4 – 2.2.5 for chambers 3 to 12 until the throughflow system is running on all chambers at 1 L/min.

E.2.3 Stopping an ammonia sample run to replace acid traps or perform other measurements

You will need to stop the ammonia sample run to (1) replace the acid traps, (2) perform greenhouse gas sampling, or (3) to complete your sampling. Typically, you will perform both 1 and 2 at the same time to minimise the period of time that the system is off for *replacing acid traps*.

- 2.3.1 To replace the acid traps you will require 24 flasks containing 80 mL of 0.02 M H_3PO_4 solution. Label the flasks as described in 2.2.1. *At high temperatures you might find large evaporative losses of the acid trap solution, if this occurs increase the volume of the acid trap to 100 mL.*
- 2.3.2 Close the flow meter valve associated with chamber 1 and note flow switch off time on recording sheet. Remove the rubber bung with glass straws from the inlet acid trap (1-in). Place used acid trap into collection tray and seal with lid/bung. Replace acid trap with the fresh acid trap labelled 1-in and replace rubber bung with glass tubes. Remove the rubber bung with glass straws from the outlet acid trap (1-out). Place used acid trap into collection tray and seal with lid/bung. Replace acid trap with the fresh acid trap labelled 1-out and replace rubber bung with glass tubes.
- 2.3.3 Slowly open the flow meter valve associated with chamber 1 until a flow rate of 1 L/min is achieved. Record on the recording sheet the time and date that the air flow was switched back on.
- 2.3.4 Repeat 2.3.2 – 2.4.3 for chambers 2 – 12 until all acid traps have been replaced.

E.2.4 Preparing acid trap samples for analysis

The acid traps provide a cumulative sample of NH_3 losses from the individual chambers. However, the laboratory analysis is conducted on a liquid sample and reported in mg N L^{-1} , therefore it is necessary to make the entire acid trap up to a known volume before submitting a subsample of the solution to the laboratory.

- 2.4.1 Prepare sample vials for submission to the North Wyke laboratory, assign each vial with a North Wyke (NW) experimental number, followed by a unique number for each sample (eg NW743/B001). To ensure sample IDs are not washed off use the label printer in the Selborne building to identify your samples. Along with the 24 acid trap samples, submit two samples of the H_3PO_4 solution used in the acid traps. (NB: the outlet acid traps should be submitted for analysis because they contain the NH_3 emitted from the treated chambers. Submission of the inlet acid traps is optional depending on your experiment objectives).
- 2.4.2 Using a funnel, transfer the entire acid trap sample into a 100 mL volumetric flask, rinse the flask with DI water and transfer washings to the 100 mL volumetric flask. *Take care not to overfill the volumetric flask beyond the 100 mL mark.* Using a

disposable pipette make the solution up to 100 mL (ie the volume line on the flask), mix this solution thoroughly with several inversions.

2.4.3 Decant a subsample of this solution into a 30 mL vial for submission to the lab, dispose of the remaining sample. Rinse the volumetric flask and funnel a minimum of three times before using again.

2.4.4 Repeat 2.4.2 – 2.4.3 for each acid trap sample.

E.2.5 Submitting samples for analysis

Solution samples are submitted for analyses using automated colorimetry. This analysis is typically conducted by Liz Dixon and he should be liaised with, to notify her of sampling dates and numbers. An analysis request form must also be completed and sent to nw.sampleregistration@rothamsted.ac.uk. See shared drive for example registration form.

Appendix F Nitrous Oxide Gas Sampling Protocol (used in chapter 2)

Nitrous oxide flux measurements from soils using static chambers are made by measuring the increase in the headspace N₂O concentration within a covered, air-tight chamber over a 60 min period.

F.1 Chamber

Equipment and checks:

- Chambers – check there are no cracks and that all chamber ports are sealed/plugged.
- Chamber lids – check that gas sampling tube and valve are attached, and that sample tube is not squashed or kinked.

F.2 Sampling

Equipment and preparation:

- Labelled *evacuated* gas sampling vials in polystyrene rack (x 51).
- 30 ml syringes filled with fresh ambient air (collect this from outside) x 48 (4 syringes of replacement air needed for each chamber (x 12), at each sampling (x 4) occasion.
- 30 ml syringe x 12 for taking gas samples.
- Wide bore (green; 0.8 mm ID) and narrow bore (orange; 0.5 mm ID) syringe needles.
- Timer
- Recording sheet

Before and after the sample run note the controlled environment temperature. Place lids on all chambers and conduct the chamber sampling run (see F 2.2 below).

Sampling strategy:

For a 12-chamber experimental system, with sampling times of 0, 20, 40 and 60 min you need to devise a sampling strategy which allows you to sample all 12 chambers and return to chamber 1 before 20 min has passed. When learning the technique, you might want to allow 1 min between each chamber's sample being collected, for example sampling times see table F-1 below:

Table F-1: Example sampling strategies for gas sampling of 12 chambers.

Chamber number	Sample collection time (mm:ss; with 1 min between chambers)				Sample collection time (mm:ss; with 1:15 min between chambers)			
	0 min sample	20 min sample	40 min sample	60 min sample	0 min sample	20 min sample	40 min sample	60 min sample
1	00:00	00:00	00:00	00:00	00:00	20:00	40:00	60:00
2	01:00	01:00	01:00	01:00	01:15	21:15	41:15	61:15
3	02:00	02:00	02:00	02:00	02:30	22:30	42:30	62:30
4	03:00	03:00	03:00	03:00	03:45	23:45	43:45	63:45
5	04:00	04:00	04:00	04:00	05:00	25:00	45:00	65:00
6	05:00	05:00	05:00	05:00	06:15	26:15	46:15	66:15
7	06:00	06:00	06:00	06:00	07:30	27:30	47:30	67:30
8	07:00	07:00	07:00	07:00	08:45	28:45	48:45	68:45
9	08:00	08:00	08:00	08:00	10:00	30:00	50:00	70:00
10	09:00	09:00	09:00	09:00	11:15	31:15	51:15	71:15
11	10:00	10:00	10:00	10:00	12:30	32:30	52:30	72:30
12	11:00	11:00	11:00	11:00	13:45	33:45	53:45	73:45

F.2.1 Ambient air sampling

- 2.1.1 Make two needles ready, one green, and one orange, by placing them into the polystyrene rack, prepare a duplicate set of needles in case they are needed.
- 2.1.2. Charge/discharge syringe several times expelling any contaminants.
- 2.1.3. Head outside and find a location away from any emission sources (eg Tennis courts). Working at shoulder height charge and discharge syringe several times expelling any contaminants and fill the 48 syringes with 30 mL of “fresh air” and store in rack ready for use in the gas sampling.

- 2.1.4. Again, sample fresh air (as described in 2.1.3) by filling the sampling syringe to 30 mL, attach the green needle onto the syringe, push needle through the appropriate vial seal and depress plunger to pressurise the vial (vials are marked BG for background samples).
- 2.1.5. Holding plunger down to keep the vial pressurised, insert orange needle through vial seal and fully depress plunger.
- 2.1.6. Excess gas will escape via the orange needle making a hissing noise. When the pressure equalises the noise ceases, then you can remove the orange needle, replacing it in the rack.
- 2.1.7. Withdraw syringe with green needle from the vial, remove needle from the syringe and replace in the rack.
- 2.1.8. Repeat from step 2.1.4 to 2.1.7 until three background samples are completed.

F.2.2 Sample run: placing lids on chambers and taking gas samples

Samples are taken from each chamber after the lid has been placed on top at 0, 20, 40 and 60 minutes.

- 2.2.1 Make two needles ready, one green, and one orange, by placing them into the polystyrene rack.
- 2.2.2 Before sampling charge/discharge the syringe several times expelling any contaminants.
- 2.2.3 Using the syringe flush air through the tubing on the top of all chamber lids, thus removing any contaminants or water, etc. Leave syringe empty and attached to valve.
- 2.2.4 Ensure all chamber ports are plugged except the port which will be used to replace sampled air with the fresh air collected prior to sampling. Attach syringe containing replacement fresh air to port on side wall.
- 2.2.5 Place the lid on the chamber and seal it with tape, ensure it is on flat and note the run start time. Start the timer and fill the syringe to 30 mL by drawing gas from the chamber into the syringe, shut the valve so the tubing/chamber is sealed.
- 2.2.6 Pull the syringe off the valve and open the valve on the replacement air port.
- 2.2.7 Attach the green needle to the syringe, inject the sample into the appropriately labelled vial as in 2.1.4-2.1.7 above.

- 2.2.8 Fully depress the replacement air syringe and close the associated valve, leave the replacement air syringe attached for now.
- 2.2.9 Move on to the next chamber and repeat the process, placing the lid on the chamber typically 60 or 75 seconds from the previous lid being put in place (see sampling strategy table). This time can be increased/decreased depending on the sampler's speed in sealing the chambers.
- 2.2.10 Repeat 2.2.1 to 2.2.9 until all lids are in place on the chambers.
- 2.2.11 Return to chamber number one. Remove all empty replacement air syringes and replace with full (to 30 mL) syringes.
- 2.2.12 The second sample must be taken when the lid has been in place for 20 minutes, the third when the lid has been in place for 40 minutes, and the fourth when the lid has been in place for 60 minutes.
- 2.2.13 Before sampling charge/discharge the syringe several times expelling any contaminants.
- 2.2.14 Attach empty syringe to valve and open valve allowing gas movement from chamber to syringe.
- 2.2.14 At the appropriate sampling time (20, 40 or 60 minutes after the lid was placed on the chamber) draw gas from the chamber and fill the syringe to 30 mL.
- 2.2.15 Pull the sample syringe off the valve and open the replacement air valve.
- 2.2.16 Attach the green needle to the sample syringe, inject the sample into the appropriately labelled vial as in 2.1.4-2.1.7 above.
- 2.2.17 Fully depress the replacement air syringe and close the valve.
- 2.2.18 Move on to the next chamber and repeat the process, typically leaving 60 or 75 seconds between sampling 2 chambers.
- 2.2.19 Repeat steps 2.2.11-2.2.18 for the 40- and 60-minute samples.
- 2.2.20 After completing all sampling remove the chamber lids and the empty syringes which can be re-used on the next sampling occasion following thorough flushing with fresh air.

F.2.3 Submitting samples for analysis

Gas samples are submitted for analyses via gas chromatography. This analysis is typically conducted by Neil Donovan and he should be liaised with, to notify him of sampling dates

and numbers and to also be provided with vials for gas sampling. An analysis request form must also be completed and sent to nw.samplerregistration@rothamsted.ac.uk. See shared drive for example registration form.