Strategies to improve nitrogen use efficiency and yields in UK forage crops

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ABSTRACT

Grasses and lucerne are the main crops of choice for dried forage crop growers in the UK, who use vegetative forage tissue to formulate bulk animal feeds. To maximise yield, the forage crop industry uses substantial fertiliser applications, however not efficiently, which is directly related to limited research into forages. Efficiency must be increased throughout the industry; therefore this thesis aims to investigate several strategies to increase yield and efficiency of UK forage crop production.

Firstly, a range of nitrogen status marker genes for use in forage crops were validated in the laboratory. Testing of field samples showed a link between soil conditions and future yields. This provides knowledge platforms that can be used by growers to ensure adequate, but not excessive, fertiliser use.

Soil nitrate availability to forage crops was tested with a new method using soil columns in conjunction with nitrate-selective sensors. This method provided extensive data of the soil nitrate profile in columns, and showed to be superior to techniques in current published literature. The data collected with the use of soil columns and nitrate-selective sensors was then used to investigate management practices, including how defoliation and intercropping can affect soil profiles.

Next, the potential of the biostimulant fulvic acid to increase vegetative yield in forage crops was investigated. It was found that the treatment with fulvic acid resulted in vegetative yield increases in numerous lucerne cultivars, across a range of growth conditions. Furthermore, it was observed that nodulation and microbial growth was also affected by fulvic acid treatment. To assess the mode of the action that determines biomass increases transcriptome analysis was undertaken, which suggested fulvic acid may be a viable biostimulant for lucerne, providing yield increases without significantly higher inputs.

Taken together, this research provides a great resource of information to aid growers in producing high quality forage more efficiently.

"*I know I've got a degree. Why does that mean I have to spend my life with intellectuals? I've got a life-saving certificate but I don't spend my evenings diving for a rubber brick with my pyjamas on.*" - Victoria Wood

Oxford Dictionary of Quotations by Subject (ISBN 0199567069)

TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

LIST OF COMMON ABBREVIATIONS

BAGCD	British Association of Green Crop Driers
BLAST	Basic local alignment search tool
CFU	Colony forming unit
DE	Differentially expressed
DEFRA	Department for Environment, Food and Rural Affairs
dH_2O	Distilled water
DM	Dry matter
EIN	Early initiating nodule
FA	Fulvic acid
FAME	Fatty acid methyl ester
GO	Gene ontology
HS	Humic substances
ICP	Ion-coupled plasma
LV	Lytic vacuole
MC	MFA control
MDS	Multi-dimensional scaling
MFA	MPXA FA
MS	Mass spectrometry
MSA	Multiple sequence alignment
N	Nitrogen
N_2O	Nitrous oxide
NA	No application
NH_4^+	Ammonium
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NO_3^-	Nitrate
NUE	Nitrogen use efficiency
NVZ	Nitrate vulnerable zone
OD	Optical density
PCA	Principle component analysis
PLFA	Phospholipid fatty acid
PM	Pink nodule
PSV	Protein storage vacuole
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative reverse transcription PCR
RNA-seq	RNA-sequencing
RT	Room temperature
UAA	Utilised agricultural area
VC	VFA control
VFA	Vitalink FA
WN	White nodule

Chapter 1: Introduction

1.1 THE IMPORTANCE OF FORAGE CROPS

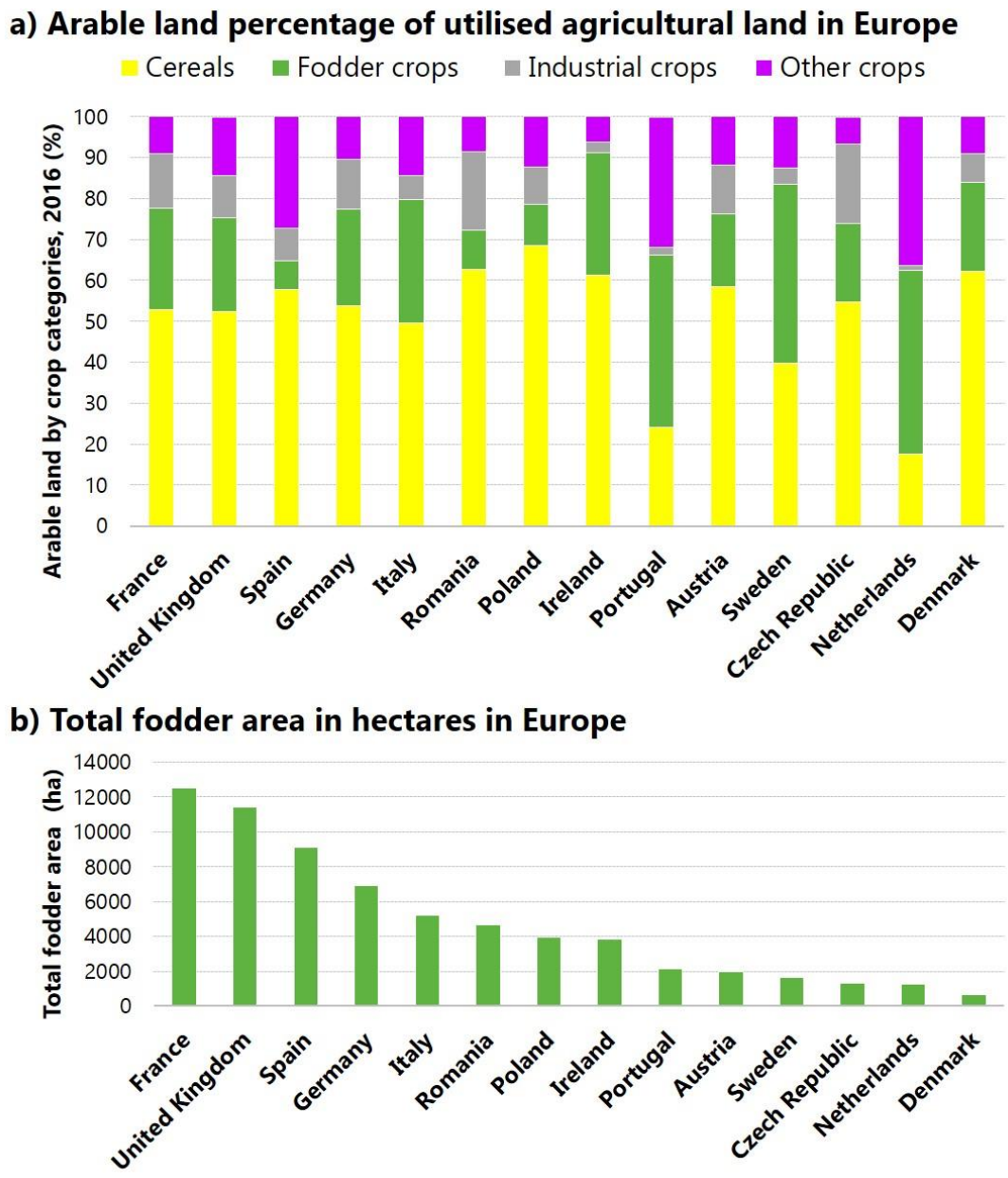
Forage crops are grasses, legumes, root crops, and trees, grown globally for animal feed. Their cultivation provides the necessary bulky feed for the animal product industry for human consumption. In this section the relevance of these crops in Europe and the UK will be discussed.

1.1.1 Temperate crops and cultivars

Forage grasslands represent 26 % of global land area, and 70 % of agricultural land [1]. These grasslands are used to feed livestock with forage crops available for tropical and temperate climates. In the UK, temperate forage crops are cultivated, and these are usually grasses (*Poaceae*) or herbaceous legumes (*Fabaceae*). In temperate climates like the UK, the main forage grasses include ryegrass (*Lolium* spp.), fescue (*Festuca* spp.), bentgrass (*Agrostis* spp.), and orchard grass (*Dactylis* spp.), or hybrids such as *Festulolium* [2; 3]. The most commonly cultivated legumes are medics (*Medicago* spp.), trefoil (*Lotus corniculatus*), clover (*Trifolium* spp.), and vetches (*Vicia* spp.). The globally important legume lucerne or alfalfa (*Medicago sativa*) is of particular prominence in UK forage production, even termed the 'wonder protein plant' [4], and is the most widely cultivated crop along with *Lolium*, *Festuca*, and *Festulolium* grasses. Some *Brassicaceae* and *Amaranthaceae* forage species can also be cultivated for their vegetative and root tissues.

The vast array of forages, as well as the multiple cultivars available within each species or hybrid family, means a crop can be chosen for the specific environment of cultivation. These can be cultivated as stand-alone crops, or as a mixed or intercropped system. Forage crops will either be grown as part of a permanent pasture, with little to no management influence, or as low to high intensity cultivated temporary grassland, sometimes in rotation with commodity crops. A general term used by Eurostat (the statistical directorate-general of the European Commission) is "fodder crops", which describes forage crops grown using cultivation methods in utilised agricultural area (UAA), and so not as a permanent pasture. Figure

1.1 below shows the UAA of the top forage producing countries using the fodder crops metric [5]; 1.1a shows the percentage of UAA land in these countries as fodder cropland; 1.1b shows this fodder cropland as area in hectares.



<u>Figure 1.1:</u> Fodder crop production in the top forage producing European countries.

Source of data is Eurostat [5] with online data codes: ef_lac_main and Eurostat calculations. Data is for 2016 and shows top forage producing European countries for a) fodder crop arable land percentage of utilised agricultural area, and b) the total fodder area in hectares of forage crops. Note that Italy is based on predicted data, whereas all other countries are accurate measurements.

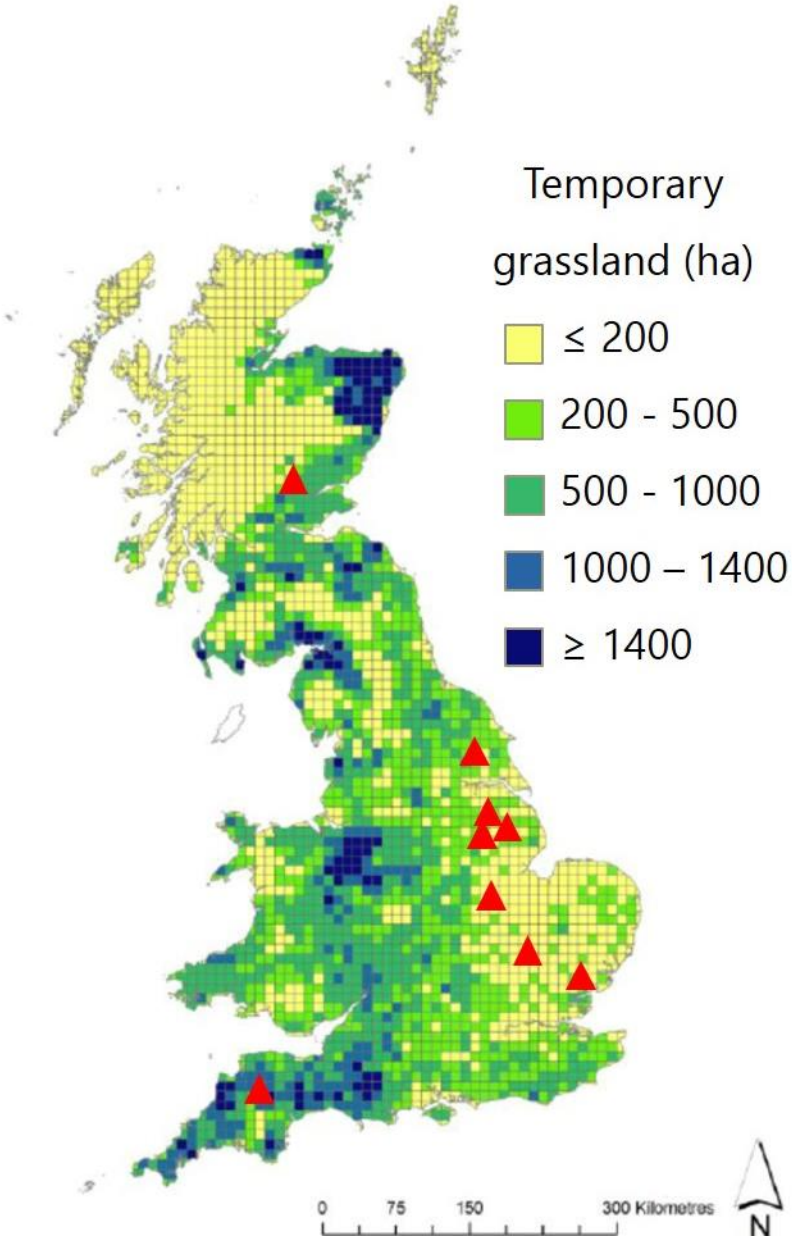
1.1.2 Relevance and cultivation in the UK

Wild forage pastures began at 9000 - 7000 BC, coinciding with the domestication of animals across North Africa, Europe, and Asia [6]. Domestication of forage species occurred from ~ 1000 BC. During the 13th - 19th centuries developments in agricultural practice and haylage production led to more forage crop cultivation. In the 19th – 20th centuries there was the rise of agricultural intensification, which was particularly prominent throughout Europe and the UK [7; 6]. Now forage crops are widespread across the UK, as shown in the temporary grassland production map of Figure 1.2. This figure shows forage production occurs across climates and soil type, including sandy soils and marginal land. This is possible as species and cultivars can be chosen to match the local climate.

Livestock can be fed directly with fresh forage crops, or with processed, partially dried or pre-digested feeds, including hay, haylage, and silage. Dried feed in the UK is predominantly produced by the British Association of Green Crop Driers (BAGCD); BAGCD produce ~ 90 % of UK dried feeds [8]. The location of BAGCD members is shown in Figure 1.2. These feeds are termed bulky feeds and are produced from grass and legume cropping, most predominantly using *L. perenne* (ryegrass) and *M. sativa* (lucerne), or a mix of these and similar grass/legume mixes. Animals can also be fed concentrates, which are generally cereals, oilseeds and legume seeds. Additionally, sugar-rich high energy feedstuffs are added to concentrates, such as molasses and fats. Using concentrates as feeds means the energy required to produce animal products is greatly increased compared to the energy input required for animal products produced from bulky feeds, with sometimes over double dry matter required per-capita yr^{-1} [9]. Moreover, these concentrate feeds for animals are in direct competition with materials for foodstuffs for human consumptions [10]. As animal production is heavily resource intensive, and concentrate food diverts land from direct food production for humans, using forage crops as bulky feed instead of cereal-based concentrate feeds is necessary to lowering feed vs. food competition. Making forage crops more efficient in their production will greatly help this aim.

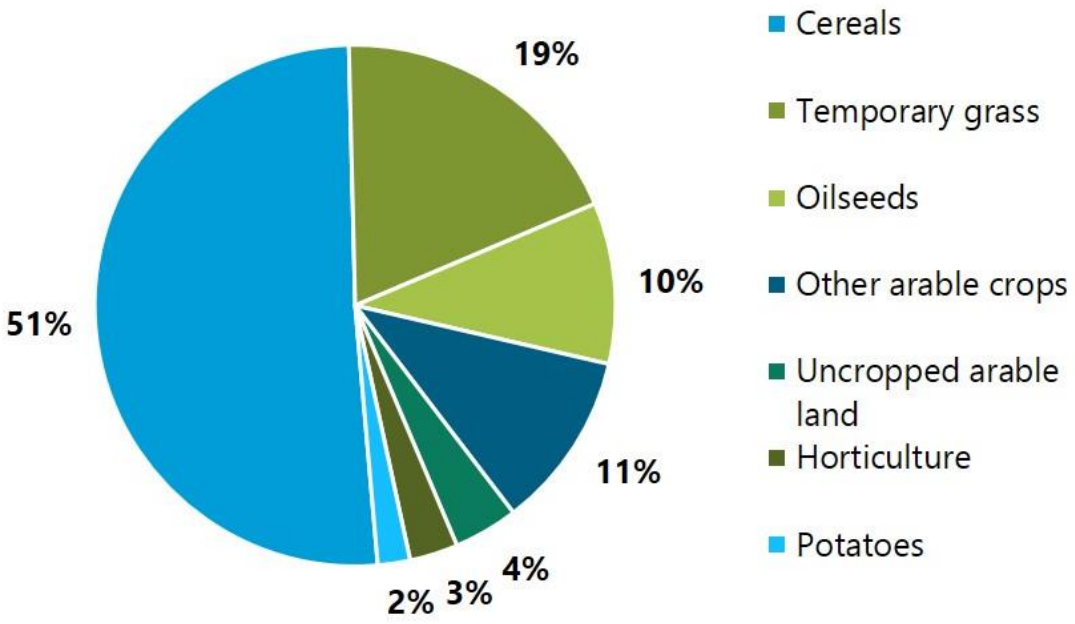
Cultivation of forages is widespread across the UK, and globally there has been a rise in many countries specific breeding programmes for forage crop species [11-15]. There are many UK and European forage breeders, especially since there has been a rise in the use of forages as energy and cover crops, and these include DSV United Kingdom Ltd., LS Plant Breeding, Nickerson, Germinal Holdings Ltd., and Limagrain UK Ltd. Designated breeding programmes are possible due to the economic importance of forage crops. This is shown in Figure 1.3 where the UAA production percentage of crops is divided by type, with temporary grass being 19 % [16], showing how intense cultivation is hugely important to UK agriculture. As forage crop cultivation is intrinsic to intensive agriculture, forage crop cultivation is essential for supporting a growing human population.

Throughout the decades, there have been periodic developments which have intensified forage cultivation and the regulation of the industry has undergone many changes. My review entitled 'UK dried forage production: a review of industry changes and assessment of prospects for both policy and science' can be found in Appendix E. This review details the recent changes in UK forage crop cultivation, particularly those in the dried forage industry relating to BAGCD members. Most BAGCD forage crops are grown in temporary grassland, which are shown in Figure 1.2 as area of UAA. This production is high intensity compared to permanent grasslands, with high nitrogen (N) fertiliser applications and intense cutting regimes.



<u>Figure 1.2:</u> Temporary grassland area in the UK.

Map shows grassland area in hectares produced in the UK as temporary grassland, reproduced and adapted from ADAS NNFCC project 08-004 report [17]. The key shows the area in hectares of grassland per unit square, with yellow depicting less than 200 ha and dark blue as more than 1400 ha. Red triangles show the sites of British Association of Green Crop Driers (BAGCD) farms.



<u>Figure 1.3:</u> Utilised agricultural area (UAA) of UK crop types in 2018.
The percentages of UK crop types are adapted from the DEFRA Farming Statistics 2019 [16]. Forage crops include the 'Temporary grass' in dark green at 19 %, but also includes a proportion of 'Other arable crops' in dark blue, and forage legumes are designated as this crop types.

1.1.3 Nutritional content and yield

Not only are there numerous species cultivated in the UK, but their nutritional content and growth can vary significantly. The value of a forage crop depends on 1) its nutritional content, including the content of complex molecules that can change the nutrient availability, and 2) the ability to rapidly increase the vegetative biomass. Herein will be described the factors effecting nutritional content and yield in forage crops; a more detailed review can be found in Capstaff and Miller (2018) [18].

Digestibility

The nutritional status of a forage crop depends upon the relative concentrations of carbohydrates, proteins and lipids. This reflects the digestibility (D-value) of a crop, and this along with trace elements, vitamins, and minerals, provides the nutritional value available for the animal on consumption. The metabolisable energy is measured in MJ / kg dry matter (DM) [19]. The following paragraphs discuss the nutritional content of forage crops in the context of digestibility.

Carbohydrates

In forage crop vegetative tissue, carbohydrates can be 50 - 80 % of DM. Carbohydrates will primarily be insoluble structural polysaccharides including cellulose and hemicellulose, or in the storage forms of starch and water-soluble sugars. The different ratios of these carbohydrates within a forage crop will alter its downstream digestibility. This is especially true if cell wall structures constrain digestion through limiting cell wall penetration by the animal's microbial populations within the gut [20]. As such, molecules like lignin, a polyphenolic compound within forage and not a carbohydrate, can bind the structural carbohydrates cellulose and hemicellulose, having a huge impact on forage digestibility [21]. Forages with decreased lignin concentration during growth will increase the digestible DM of the feed. This is true for *L.* perenne, where high soluble sugar content alongside low lignin content provides a high D-value [22].

Protein

Forage proteins in bulky feeds determine the N availability to animals. The majority of this protein, like with all land plants, will be in the form of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), although the relative amounts do vary between species and cultivars [23]. Other major N-containing compounds include N-storage proteins, nucleic acids, and nitrate [23]. Forage crops with especially high protein contents are the forage legumes, especially *M. sativa*, *T. pratense*, and *T. repens* [22]. Lignin can negatively affect the digestibility of protein, as well as carbohydrates, by binding and preventing hydrolysis and the subsequent breakdown of the molecules. Some micronutrients like condensed tannins or proanthocyanidins can affect protein digestibility, by inhibiting degradation through binding. This binding can be advantageous for feed, as binding reduces rapid protein degradation which reduces bloat [24; 25]. There is a delicate balance between N availability through protein degradation and reducing bloat for animal productivity [26]. Grasses contain little or no proanthocyanidins content, but many legumes have very high levels of up to 18 % DM [27; 26].

Lipids

Lipid content is the final factor effecting D-value in forage crops. They are mostly found as polyunsaturated fatty acids (PUFAs); PUFAs can be in the range of 10 – 30 g kg^{-1} [28] and the most abundant kinds is α-linolenic acid at ~ 62 % total lipids [29]. The PUFAs of linolenic and palmitic acid are also present in high numbers [30]. These lipids are important in both the feed composition, but also the quality of the final animal product. It has been shown that forage diets with low PUFA levels when compared to cereal concentrate diets will produce leaner meat [31; 32]. In addition, forage has been shown to produce milk with lower PUFA content and higher levels of trans-fatty acids [33; 34]. Therefore, studies have been carried out to profile PUFAs across forage species [35]. Differences in PUFA content can be seen between cultivars, species, harvest period, and environment [36; 29]. The grasses *L. perenne*, *F. pratensis*, and

Festulolium hybrids vary in their PUFA content at the beginning and end of their growth season, as well as between individual cuttings [37].

Trace Elements

Trace elements and minerals in forage crops are important for animal health. It is important that the nutritional balance of feeds is optimal for the animal, to ensure healthy processes like immunity without need for unnecessary antibiotic use. Zinc is important for immunity and, although supplements can be used to aid animal health, such additions can cause wasteful excretion [38]. In contrast, the accumulation of toxic minerals must also be avoided in forages. The required balance in trace element nutritional content is best shown with selenium as an example, where low levels are beneficial for animals and high concentrations cause toxicity [39]. Trace elements can also make the forage unpalatable for animals, and must also be considered when improving the trace element content of forage crops.

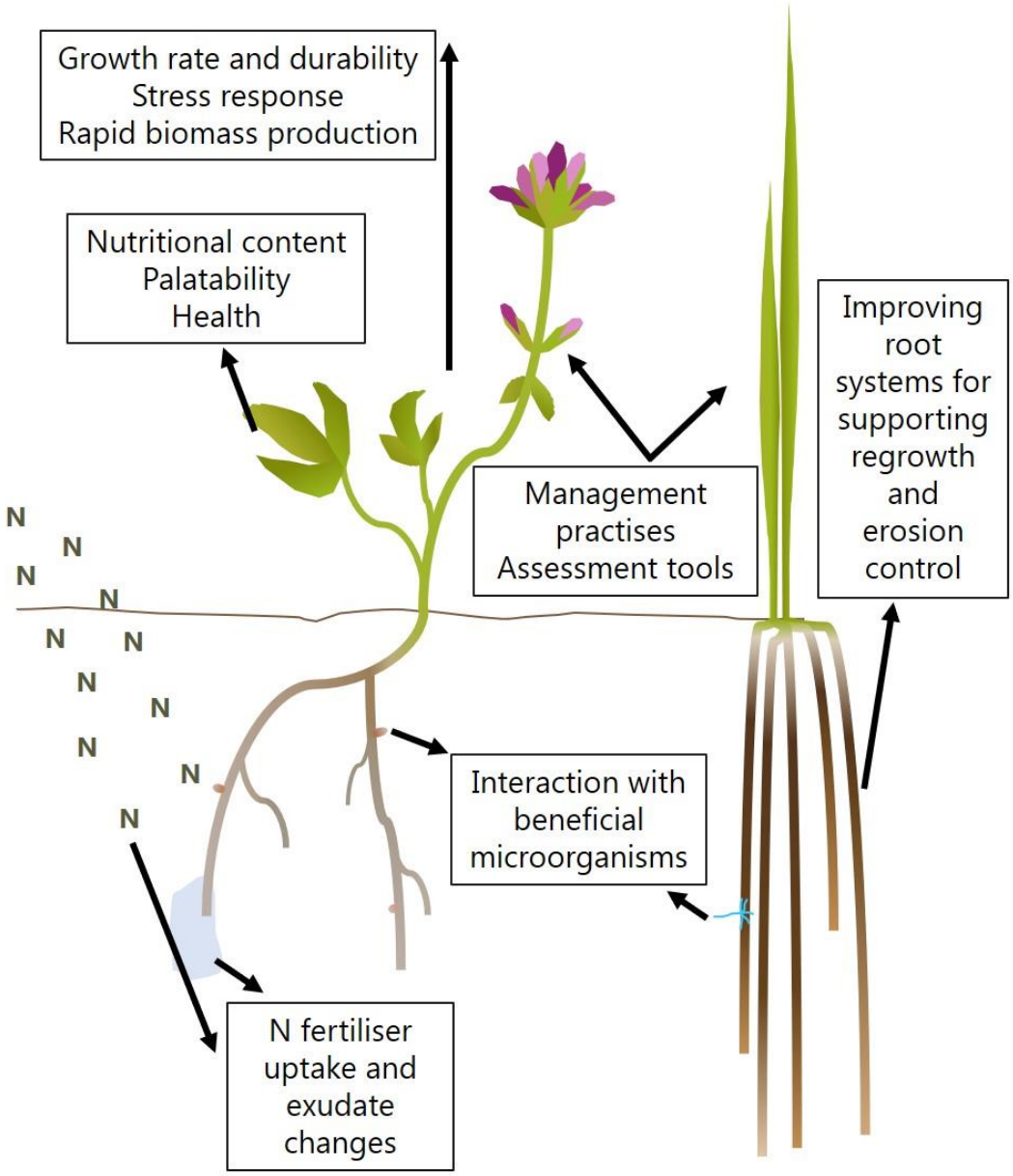
Biomass

Along with studies aligned to animal nutrition, the most important area of forage crop research is in rapid biomass production, particularly for height and vegetative biomass [40]. This is because crops are either cut or grazed directly, with high frequency. For dried forage producers such as the BAGCD, crops are cut throughout the spring/summer growing season on average 6 – 8 weeks (or 4 – 7 cuts depending on local climate, weather conditions and growth). Although many different plant species can be grown for forage, grasses are most desirable as they have a shoot meristem which promptly responds to cutting by increasing vegetative growth. In many forage grasses, the correlation of aboveground cutting or grazing has been correlated to increased shoot growth alongside increased root exudation [41-47]. These exudates cause a flush of carbon-containing compounds into the surrounding soils, which microbes can use to mobilise nutrients, which in turn will sustain the aboveground regrowth of the plant. Therefore, maintaining both nutrient and water supply for regrowth post-cutting is important in forage crop biomass production [48], and N

availability to the crop is especially important [45; 49; 50]. Strategies to improve biomass production in forage crops will be discussed in Chapter 5.

Current research in forage crop production

Despite Figures 1.1, 1.2, and 1.3 showing forage wide cultivation and importance as crops, including specific breeding initiatives for species, forage crops have been relatively forgotten in modern research when compared to cereals, vegetables and fruits [18], and their potential remains largely unexplored [51]. Techniques in genetic transformation of forage crops have been developed [52], which have made the study of individual genes possible for their effect on nutritional content. Figure 1.4 below shows some of the main targets for improvements in forage crops. Individual advances have been reviewed elsewhere [13; 53-58; 18; 59; 60], and will discussed further in Chapter 3.



<u>Figure 1.4:</u> Targets for improved forage crops.

Adapted from Capstaff and Miller (2018) [18], showing the main targets for improvement in forage crop research.

1.2 FORAGE DRYING IN THE UK – BRITISH ASSOCIATION OF GREEN CROP DRIERS (BAGCD)

1.2.1 Methodology conception

With the increase in agricultural intensification at the beginning of the 20th Century [7; 6; 18], new technologies were developed. In the 1920s drying technology gained attention [61; 62], and in the 1930s dried forage was bulk produced in the UK [63]. The process of high temperature drying established, enabled the production of pelleted feed from forage which lasted longer than normal fresh fodder, haylage or silage, whilst preserving nutritional content [64]. High temperature drying was particularly prominent post-War, where lowering waste from fresh forages was paramount to stockpiling dried feed efforts [65]. From the 1950s to 1970s, new drying technologies continued to develop, and many UK growers purchased rotary machines. These rotary machines use high temperature and rotation to dry grass and legumes for manufacture as pellets and are used by BAGCD sites to this day. Further information on the method development across Europe and the UK can be found in Appendix E.

1.2.2 Present production of dried forage

Present production locations of dried forage are shown in Figure 1.2, with sites represented as red triangles on UK map. BAGCD is an association of numerous farms based in Cambridgeshire, Devon, Essex, Hertfordshire, Lincolnshire, Perthshire and Yorkshire. In total they farm approximately 7500 hectares of land through high intensity, temporary grasslands across all these sites, with only one farm as permanent pasture. Crop rotations of forage crops are cultivated with break crops of wheat, maize or oilseed. As before, cuts are taken throughout the spring/summer growing season every 6 – 8 weeks. The crops grown by BAGCD members can be found in Table 1.1; crops which are currently cultivated are shown, alongside those recently cultivated or of interest to members.

Table 1.1: Forage crops cultivated in the UK by the British Association of Green Crops Driers (BAGCD).

Crops are shown which are currently cultivated, or are of interest to BAGCD for future cultivation. Crops are split for grasses and legumes, with species and common names included.

Current crops cultivated by BAGCD

Grasses		**Legumes**	
Species	**Common name**	**Species**	**Common name**
Lolium perenne	Perennial Ryegrass	*Medicago sativa*	Lucerne/Alfalfa
Festuca arundinacea	Tall Fescue	*Lotus corniculatus*	Birdsfoot Trefoil
Festuca pratensis	Meadow Fescue	*Trifolium pratense*	Red Clover
Festuca x *Lolium* (various)	*Festulolium*	*Pisum sativum*	Spring Pea
Phleum pratense	Timothy grass		
Anthoxanthum odoratum	Sweet Vernal grass		

Crops of interest to BAGCD

Grasses		**Legumes**	
Species	**Common name**	**Species**	**Common name**
Dactylis glomerata	Cocksfoot	*Onobrychis viciifolia*	Sainfoin
Lolium x boucheanum	Hybrid ryegrass	*Trifolium repens*	White Clover
Lolium multiflorum	Italian Ryegrass	*Galega orientalis*	Forage Galega
Lolium westerwoldicum	Westerwold Ryegrass		

1.2.3 The main difficulties associated with the forage crop industry is nitrogen use efficiency

The industry faces many challenges at present, ranging from high fuel costs and support payment changes, to soil erosion and biodiversity initiatives. Again, these are discussed in Appendix E, where industry, policy and research are detailed in parallel, and discussed regarding the operations of the BAGCD. Undoubtably one of the most prominent challenges felt by BAGCD members is in future N fertiliser use.

In the highest forage producing countries in Europe, N use efficiency (NUE) of fertilisers for all crops is variable, with NUE low in the UK, shown in Figure 1.5. It is important to define NUE and how it can be measured. The definition of NUE has been used sporadically and inaccurately across the years, with limited agreement between studies throughout the decades [66-71; 18]. This is due to the complicated nature in which N acts within the plant system. In reviews by both Xu et al., (2012) [72] and Good et al., (2004) [73] the authors highlight that efficiency can be monitored using different parameters. Some definitions are based on grain yield and others are for biomass, with only the latter relevant to forage crops. Table 1.2 is an amalgamation of the definitions as set out in these papers as well as my own contribution.

Some definitions are interested in the N distribution processes from the environment through the plant, such as nitrogen uptake efficiency (NupE), nitrogen transport efficiency (NTE), and nitrogen remobilization efficiency (NRE). These primarily measure the mechanisms by which the N can be made available from the soil supply to the tissue of interest.

Other terms are associated with how biomass changes in differing conditions, such as nitrogen use efficiency (NUE), nitrogen harvest/usage index (NHI), and nitrogen physiological use efficiency (NpUE). These terms measure of how the mechanisms of each genotype give a phenotype for yield and productivity. These terms can also be adapted for the measurement of nutritional status. For example, NUE can be modified to quantify the amount of a certain compound accumulated within tissue, based on N availability.

Table 1.2: The multiple definitions surrounding nitrogen use efficiency (NUE).

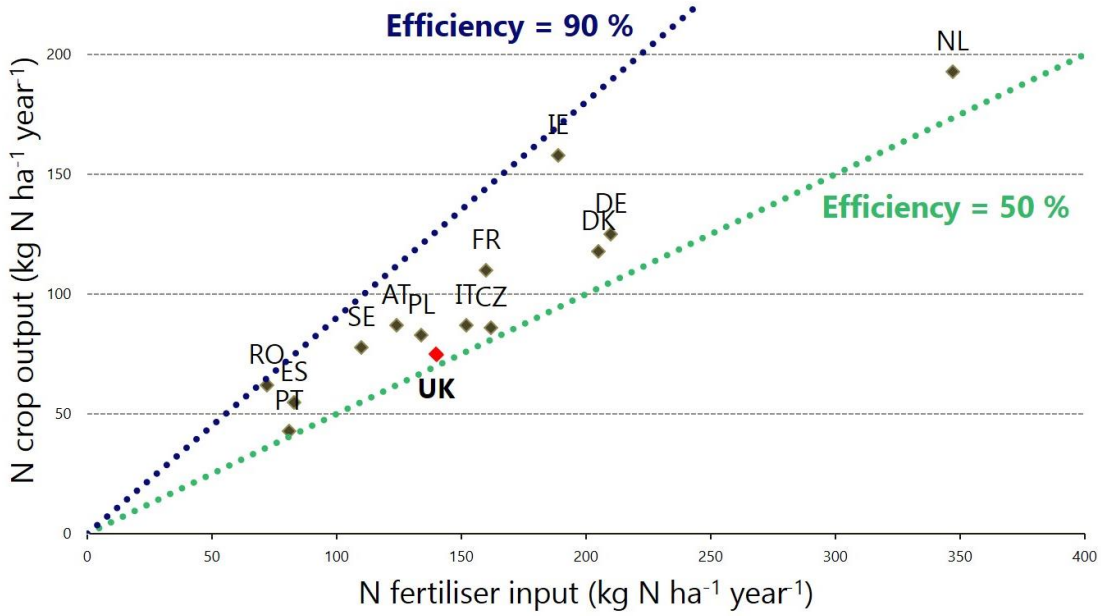
Definitions are adapted from those given in Xu et al., (2012) [72] and Good et al., (2004) [73].

Term	Equation	Definition
Nitrogen use efficiency (NUE)	Relevant biomass ÷ N available for plant	Total biomass of tissue of interest (e.g. shoots, grain) for N supplied
Nitrogen harvest/usage index (NHI)	N contents of relevant biomass ÷ N contents of whole plant	Shows N in relevant tissue whilst considering absolute biomass
Uptake efficiency (NupE)	N in plant ÷ N available	Capacity of the roots to uptake N available in soil
Utilization efficiency (NUtE)	Relevant biomass ÷ total N in plant (all tissues)	The percentage of N assimilated into the tissue of interest (e.g. grain)
Nitrogen transport efficiency (NTE)	N in above ground tissue ÷ amount of N taken up by roots	Relates how much N is transported after uptake
Nitrogen physiological use efficiency (NpUE)	Difference in relevant tissue weight with N application and without	Shows net efficiency of plant tissue with N application (e.g. fertilizers)
Agronomic efficiency (AE)	The cost difference of NpUE	The monetary difference between N application and without
Nitrogen remobilization efficiency (NRE)	N remobilized from sink tissue to source tissue	Measures how well plant redistributes N to where it is required (e.g. grain)

NUE, in terms of N fertiliser input compared to crop output, is low in the UK. Figure 1.5 shows a comparison between the NUE of all crops in the UK and the other top forage producing European countries. The UK is close to an overall NUE rate of 50 %, which greatly impacts all crop production but especially forage crops. Grasses and legumes have a slightly larger N content than cereal grain crops and therefore require higher N rates for optimal production [74]. Deficiency seriously limits forage crop production by restricting protein assembly, and primary and secondary metabolism. Forage crops have a high N demand of leaf photosynthetic tissue, and deficiencies impact many growth parameters [75]. This N requirement is exacerbated by the high cutting regimes of forages.

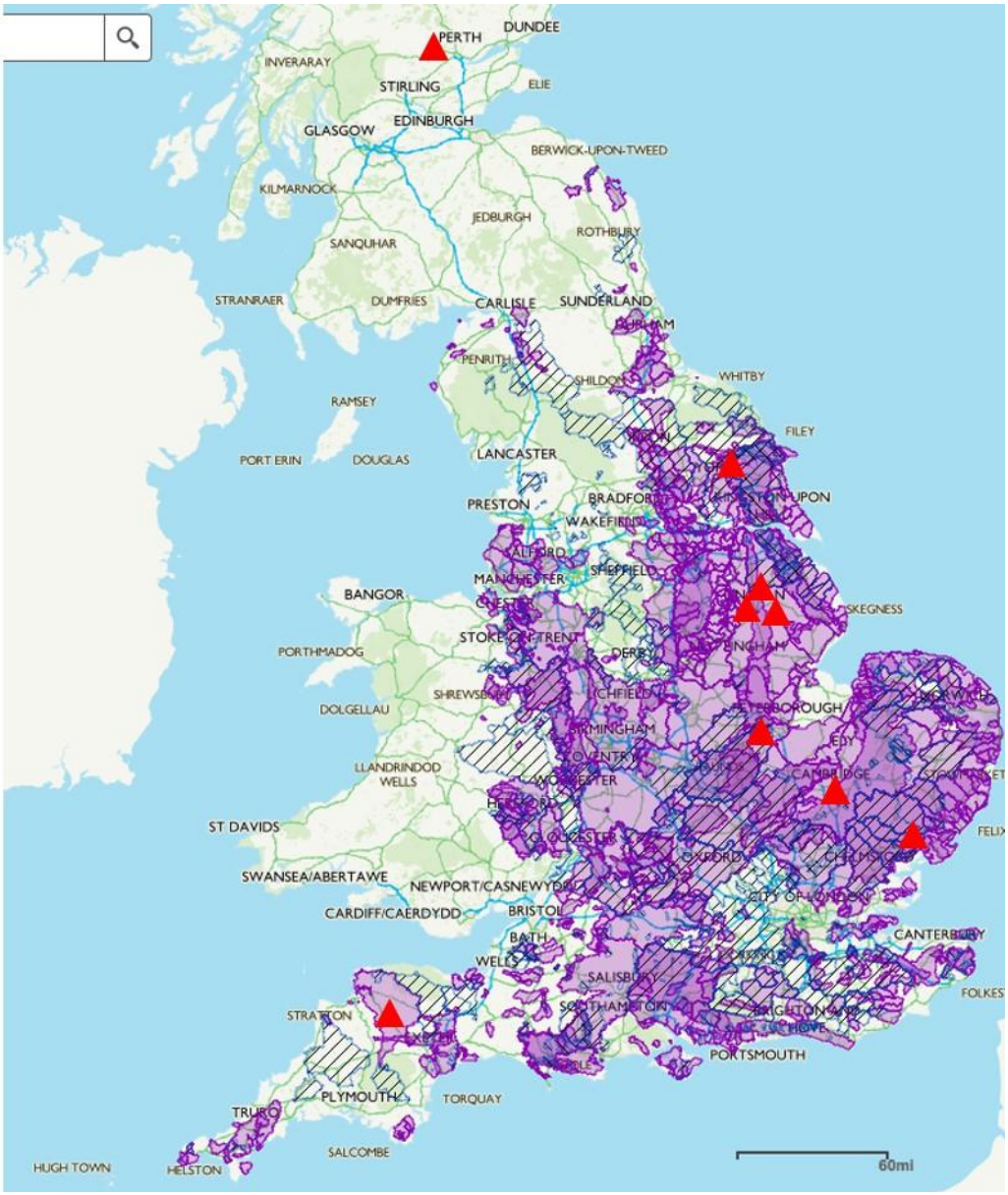
Over the past three decades there has been an increase in policy reforms to curb excessive N fertiliser use [76-79]; again details of these changes are discussed in Appendix E. Most importantly to the dried forage industry is the BAGCD exemption for N fertiliser use in the Fertiliser Manual RB209 8th addition [79]. In this exemption it is stated that BAGCD members can add up to 500 – 700 kg N ha^{-1} yr^{-1} to their crops depending on irrigation level; this application rate is significantly higher than the 2018 average N fertiliser use of UK farms for all other crops at 144 - 152 N ha^{-1} yr^{-1} [80]. BAGCD sites are found in nitrate vulnerable zones (NVZs), shown in Figure 1.6, which has confounding implications for NUE. NVZs make up approximately 58 % of the land in England, which has increased in total area from 2013 by 1300 km^2 [81; 82]. These areas are determined by land gradients, ground cover, water sources, soil types, and weather conditions [83]. In Chapter 4 such implications, as well as current assessment methods, will be explored in more detailed.

Fertiliser efficiency of all crops in highest forage producing European countries.



<u>Figure 1.5:</u> N fertiliser efficiency of all crop types in the highest forage producing European countries.

Data is for fertiliser efficiency of all crop types in 2015 for those European countries which are the top forage crop producing. Countries are shown as diamonds (using International organization for standardization United Nations coding) for N fertiliser input against total N crop output of tissue of interest (see NUE from Table 1.2). UK is depicted as red diamond. Efficiency is indicated for 50 % (green dotted line), and 90 % (blue dotted line).Source of data is Eurostat [84] with online data codes: aei_pr_gnb and Eurostat calculations.



<u>Figure 1.6:</u> Nitrate vulnerable zones (NVZs) in England.

Map shows total areas in England which are classed are nitrate vulnerable zones, reproduced and adapted from [85] Purple shows areas which are NVZs, with diagonal blocks showing areas where drinking water may be affected due to proximity to NVZs. Red triangles show the sites of British Association of Green Crop Driers (BAGCD) farms. Source: Environment Agency, UK.

1.2.4 Grassland research

As mentioned previously, forage crops can be cultivated under many different intensity systems. Forages can be grown in permanent pastures, which due to their stability are known to be important in preventing soil erosion, and contribute to fixing atmospheric carbon, with forages sequestering 34 % of global carbon stocks [86]. Permanent grasslands are capable of increasing biodiversity-richness and genetic variability [86], and provide crucial ecosystem services including water services, climate regulation, erosion prevention, cultural services, and clearly biomass production [87]. As there is no tillage of the soil, permanent grasslands can have positive effects on diversity, erosion, and emissions [88-92].

Despite the 'natural capital' [93; 94] within permanent pastures, most forage cultivation for agriculture is through high intensity, temporary grasslands, often in rotation with commodity crops. This high input/high output system has been pushed by continued global pressures for animal products [95-100]. However, there has been little research targeted to temporary grasslands, and it is imperative that we better understand temporary grasslands to determine their role in agricultural nutrient cycling when compared to permanent pastures.

Managed, temperate grasslands are of particular interest in biodiversity studies to determine how N fertiliser use of intense production can affect the environment. Significant habitat changes and declines of biodiversity in managed forage crops due to pollution from N fertilisers have been found [101]. These changes have been replicated in both short- and long-term experiments for many species [102-109]. For soil erosion, temporary cultivation may not be as beneficial as permanent pastures as more complex root architecture may not have had a chance to develop [110]. Moreover, many forage crops used in intensive cultivation (see Table 1.1) are associated with increased soil aggregate stability [111], and improve soil organic matter [112], when in permanent grasslands. Furthermore, emissions are known to differ with fertiliser use and the crop cultivated [113; 114], and at present there is limited data for how temporary forage cropland with intensive cutting affects this loss [115; 116].

1.3 STRATEGIES TO IMPROVE NITROGEN USE EFFICIENCY IN FORAGE CROPS

1.3.1 Project aims

It is difficult to draw parallels between permanent grassland research and temporary forage cultivation for dried bulk feed due to a lack of studies aimed specifically at the industry. Forage crops are grown as feed for animal production, and the social, environmental, and economic importance of forage in the UK has been discussed in Capstaff and Miller (2018) [18]. For UK forage growers, having ways to increase the crop's NUE is a primary focus at present. This project concentrated on three strategies for achieving improvements in NUE, in a multifaceted approach. This included assessing the plant itself during growth periods, evaluating the soil changes in forage crop production, and a possible management practice to increase yield with fewer nitrogen inputs. These were conducted in-line with current BAGCD production methods and management practices. Such a project not only tackles improving NUE in forage crops, but also provides valuable insights into temporary, high intensity legume and grasslands.

The overall goal of this project was to learn more about the basic connection between input and biomass in a range of forage crops. The main over-arching theme was to develop ways to test this relationship with the aim of increasing biomass with lowered inputs in the future. Moreover the project was aimed at providing more evidence for farmers regarding their N fertiliser use in the context of the above goal.

1.3.2 Thesis overview, contents, and hypotheses

Chapter 2: Materials and methods

All materials and methods for the thesis are described for Chapters 3 – 6 within this section.

Chapter 3: Marker genes in Festulolium can be used to assess crop and soil N status, to aid with grower decisions to improve future yield

At present farmers cannot reliably determine the N status of their crops [18]. This is predominantly hindered by unreliable and unreproducible tests for soil N levels. Presently farmers take limited samples across their growing area in the hope that this is representative of the N in the whole plot, and moreover through the whole growing season. If instead the farmer could determine their crop N status directly, then they could make a more informed decision as to how they should subsequently treat the plot.

Many genes have been shown to link N availability and expression across many crops [117], but a suite of suitable candidates which could be tailored for this need is lacking. Also, as there is a diverse variety of species and cultivars grown for forage, such a suite of genes would require high conservation. A detailed review of such potential genes available for testing is found in Chapter 3.1 for the following hypothesis:

Hypothesis for Chapter 3: Expression of N status genes is reliably related to crop production and soil N status.

A suite of N status marker genes for forages was investigated to predict crop quality and soil N status. This was developed in glasshouse from model plants to forage crops and then be applied to the field in *Festulolium*.

Chapter 4: Soil sensors can detect N profile changes under forage crops with different management regimes

Numerous management regimes have effects on grass production [118], but their effect on soil N profiles have not been formally tested for UK forage growers. These management practices include nitrate (NO_3^-) application, defoliation, and cropping method[119]. It is unknown how these practices affect the soil profile around forage crops, and a detailed review is found in Chapter 4.1 for the following hypothesis:

Hypothesis for Chapter 4: Using nitrate-selective sensors in soil columns with grass and lucerne will provide valuable data on plant-soil interactions of management practices.

To assess N profile changes we developed nitrate-selective sensors to track NO_3^- movement through the soil profile for use alongside conventional testing, in soil column systems. The effect of management practices on the forage crop *L. perenne* and *M. sativa* was then measured with the use of these sensors.

Chapter 5: Fulvic acid increases vegetative growth in the forage legume *Medicago sativa*, and is associated with influencing microbial activity

The investigation of potential biostimulants is of great interest in current plant science [120]. A potential application of a humic derivative called fulvic acid (FA) could improve forage crop production [121; 122]. Such an improvement could lower N fertiliser inputs. FA is a reasonably uncharacterised mixture of chemicals, and although it has been shown to increase growth parameters in many species [123; 124], the mode of action is unclear. Additionally, many studies lack correct controls in their investigations, as discussed in Chapter 5.1 in more detail for the following hypothesis:

Hypothesis for Chapter 5: Application of the commercially available biostimulant fulvic acid (FA) improves forage crop production.

To test this, commercially available FA was investigated for its potential to increase yields in the forage crops. Experiments in the glasshouse and field, with the goal to increase vegetative biomass in crops were conducted, with care to include nutritional controls so far unseen in the literature.

Chapter 6: Transcriptome analysis shows preferential enrichment of nodulation regulation and signalling-related genes in *Medicago sativa* following fulvic acid application

Following plant and microbial investigations in Chapter 5, there was evidence that FA was affecting nodulation in *M. sativa* (see Chapter 5.3). To aid in establishing if this link was true, *de novo* transcriptome analysis of both shoot and root tissue using RNA-sequencing was undertaken

following three days of FA application. A review of RNA-sequencing as a technique is found in Chapter 6.1 with the following hypothesis for fulvic acid:

Hypothesis for Chapter 6: RNA-seq will provide evidence of early differentially regulated genes in either shoot or root tissue upon fulvic acid application.

Expression analysis was performed for *M. sativa* following FA application to elucidate the growth increases investigated in the previous chapter.

Chapter 7: General discussion

The results of Chapters 3 – 6 will be summarised in regard to current literature and agriculture.

1.3.3 Contributions to thesis

All experiments in this thesis were conducted by me (N.C.) unless otherwise acknowledged. An undergraduate year-in-industry student, Freddie Morrison (F.M.) from Manchester Metropolitan University contributed to this work under both mine and Prof Miller's supervision. He received specialist supervision from Dr Michael Stephenson, Dr Lionel Hill, and Dr Paul Brett (all Metabolic Biology, JIC). Analysis was also performed by technical staff at both University of East Anglia (Norwich, Norfolk) and Rothamsted Research (Harpenden, Hertfordshire); most notably Dr Juan Carlos Muñoz-Garcia performed NMR with supervision from Prof Yaroslav Khimyak (both School of Pharmacy, University of East Anglia).

My industry collaborators BAGCD have supported this project with seed provision and specialist agricultural knowledge. Some sites have also provided sites for field trials. Specifically, Blankney Estates Ltd. (Blankney, Lincolnshire) have not only assisted in field trials through 2018 - 2019, but moreover samples were analysed to industry specifications at their laboratory British Chlorophyll Company Ltd.: technical assistants were Abigail Ewen, Sam Carruthers, and Hollie Compton. Field trials were performed in 2017 at Dengie Crops Ltd. (Southminster, Essex) with help from their agronomist Andrew Spackman (Farmacy Plc., Dorrington,

Lincoln), and in 2018 at both Blankney Estates Ltd. and A Poucher and Sons (Bardney Dairies) Ltd. (Market Rasan, Lincolnshire). I was also grateful to receive seed resources from Mr Ianto Thomas and Dr Danny Thorogood from the Genebank at The Institute of Biological, Environmental and Rural Sciences (Aberystwyth University, Aberystwyth, Wales).

Bioinformatic support was also provided from two Postdoctoral specialist onsite; Marker gene analysis from Dr Alexander Calderwood (Crop Genetics, JIC) and RNAseq analysis from Dr Jitender Cheema (Computational and Systems Biology, JIC).

Contributions of all above collaborators are acknowledged in Chapter 2. All experimental work performed by others and incorporated into this thesis is appropriately and fully acknowledged in the legends pertaining to display items (Figures and Tables).

Chapter 2: Materials and methods

TO NOTE

Throughout this chapter most chemicals used were from Sigma-Aldrich®, a subsidiary of Merck KGaA©, Darmstadt, Germany. For ease in the text if a chemical is available from Sigma-Aldrich then only its catalogue number will be provided. If a chemical was sourced elsewhere then this will be clearly indicated within the text.

Any reference to 'dH_2O' stands for sterile deionised water, and 'EtOH' and 'MeOH' are ethanol and methanol respectively. The abbreviations 'µL' and 'mL' have been used for microlitre and millilitre respectively to avoid any confusion with the number 1.

As outlined in 1.3.3 any contributions from collaborators is credited in the below methods; for clarity Nicola Capstaff is abbreviated to N.C. and Freddie Morrison to F.M.

2.1 N STATUS MARKER GENES

2.1.1 Arabidopsis growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) was provided by Yi Chen (Metabolic Biology, John Innes Centre). Seeds were surface sterilised with EtOH 70 % (v/v) and grown on 100 mm^2 plates (R & L Slaughter Ltd., Upminster, UK) with the following modified Murashige and Skoog (MS) medium of pH 5.7 and 1 % agar (Formedium™, AGA03): ½ MS Basal Salt Micronutrient Solution (M0529), 10 % MES buffer (M2933), 1 x Gamborgs vitamins 1000 (G1019), $MgSO_4.7H_2O$ 0.75 mM (M1880), $CaCl_2.2H_2O$ 1.5 mM (223506), KH_2PO_4 62.5 mM (P9791), with either KNO_3 (P8291) or $Ca(NO_3)_2$ (C2786) as N source at 0.3, 0.6, 3, 6, 10, 30 mM. Cation influence was negated with molarity balanced with KCl (P5405) or $CaCl_2.2H_2O$ (C3306) respectively. These media plates were then termed 0.3, 0.6, 3, 6, 10, 30 mM KNO_3/$Ca(NO_3)_2$ treatments. Seeds plates were vernalised for two days at 4 - 6 °C before being transferred standing vertically to a controlled environment room (CER) with temperature controlled at 23 °C and a photoperiod of 16 h light (90 µmol m^{-2} s^{-1}) and 18 h dark. Plants were grown for 3 weeks before tissue RNA extractions.

2.1.2 RNA extraction

20 x shoot samples were flash frozen in liquid nitrogen in 1.5 mL Eppendorf tubes. Samples were stored at -80 °C until required for RNA extractions. Frozen samples were ground using pellet pestles (Z359947). RNA was extracted using 1 mL TRI Reagent (93289) per 100 mg tissue on ice with 150 µL 1-Bromo-3-chloropropane (B9673) and shaken vigorously for 15 s. Following incubation on ice for 10 mins, to decrease DNA contamination the extraction was centrifuged at 12 000 g at 4 °C for 10 minutes. RNA from the aqueous phase was precipitated with 400 µL isopropanol (563935) and 400 µL of High Salt Precipitation solution: 0.8 M sodium citrate (71497) and 1.2 M NaCl (S3014). Samples were vortexed at high speed for 10 s, incubated at room temperature (RT = ~ 23 - 26 °C) and

centrifuged at 12 000 g at 4 °C for 15 mins. The supernatant was discarded, and the pellet washed with 1.5 mL EtOH 70 % (v/v). Samples were air-dried at RT for 5 mins and contaminated DNA removed using RNase-Free DNase Set (QIAGEN Ltd. 79254) according to the manufacturer's instructions.

RNA quality was measured on a NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific, ND-2000) for a A260/A280 ratio of ≥1.7, and concentrations were recorded. Samples were stored at -80 °C until required for cDNA synthesis.

2.1.3 cDNA synthesis

cDNA was synthesised in 22 µL final volume reactions with 100 - 200 ng mRNA using SuperScript™ II Reverse Transcriptase (Invitrogen™ 18064022, Life Technologies Ltd.) performed according to the manufacturer's instructions with oligo-dT, dNTPs and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen™ 18418012, 10297018 and 10777019 respectively, Life Technologies Ltd.), and provided 5X first-strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), and 100 mM DTT. Negative controls for qRT-PCR were also carried out with a no template control (NTC) and a no transcriptase control (NRT). cDNA was stored at -20 °C until use.

2.1.4 Candidate gene identification and primer design

A literature search produced a list of potential N status markers in *A. thaliana*, to be tested as genes of interest in samples. Genes were as follows; *CLCa* (AT5G40890), *NIR* (AT2G15620), *GLN1* (AT5G37600), *GLN2* (AT5G35630), *VSP1* (AT5G24780), *VSP2* (AT5G24770), *TIP1.1* (AT2G36830), *TIP1.2* (AT3G26520), *TIP3.1* (AT1G73190), *TIP3.2* (AT1G17810), *NADH-GOGAT* (AT5G53460) and *RBCS2B* (AT5G38420), and reference genes *ACTIN8* (AT1G49240) and *CYP5* (AT2G29960). Primers were either taken from published work, or designed in Primer3 [125; 126] and checked for properties such as hairpin loops in OligoCalc

[127]; primers used can be found in Table 2.1. Expression was confirmed in vegetative tissue including leaves and shoot apex using the Arabidopsis eFP Browser 2.0 [128-130] found in Figure A1, Appendix A.

2.1.5 Quantitative RT-PCR

cDNA was diluted 1:50 for use in quantitative RT-PCR (qRT-PCR) with SYBR® Green JumpStart Taq ReadyMix (S4438) in 20 µL reactions on 96-well plate (4titude Ltd., 4TI-0741_50). Each reaction was performed as below on ice as follows: 10 µL SYBR ReadyMix, 3 µL cDNA, 5 µL dH_2O, and 1 µL of both Forward and Reverse primer (5 µM).

Biological reactions were aliquoted in technical duplicates or triplicates, with each gene of interest being testing against one or two reference genes depending on plate capacity with number of treatments. NTC and NRT reactions were also run on the plate.

Reactions were carried out using C1000TM CFX96 Touch™ Real-Time PCR Detection System (Biorad, Hercules, California, USA). The qRT-PCR program was run using CFX Manager (Biorad, Version 3.1.1517.0823) as follows: lid set to 105 °C, 3 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, followed by one cycle of 30 s at 50 °C, followed by melt curve analysis (65 - 95 °C, with increments of 0.5 °C) with a plate read throughout. Melt curves were checked before analysis, as well as any detection in NTC or NRT wells.

Expression of the genes of interest were calculated using the arithmetic mean Ct according to analysis for $2^{-\Delta CT}$ method [131]. Single threshold on CFX Manager was used to determine the arithmetic mean for 2 - 3 technical replicates of both gene of interest and a reference gene for biological samples. The $2^{-\Delta CT}$ was calculated from the difference between these means and fold changes were expressed relative to the lowest concentration NO_3^- treatment as 100 %, for either KNO_3 or $Ca(NO_3)_2$ experiments. Due to expression ranges potentially being affected by cation molarity, the overall gene pattern of expression was calculated using the geometric mean of KNO3 or $Ca(NO_3)_2$ fold change percentages for treatment mM using Excel (Microsoft® Office 2016). The geometric

standard deviation was calculated by finding the exponential value for the standard deviation of the natural logarithms fold change percentages. Graphs were made in GraphPad Prism 7 (GraphPad Software Inc.).

Table 2.1: Primers used in study for *Arabidopsis thaliana.*

Gene name	Primer name	Sequence (5' – 3')	Source
CLCa	AtCLC F	GGTGCCAAGGTTTCACAC	Nicola Capstaff
	AtCLC R	GGCGAGAAGTTTGTAACCGG	
NIR	AtNIR F	CCGATTTCACCAACTTGCCA	Nicola Capstaff
	AtNIR R	AAGAACGTCATCAGCAGGGA	
RBCS2B	AtRBCS F	ACCCATTTCTATGTGGTCAATGC	Izumi et al., 2012 [132]
	AtRBCS R	TTCACTTTCAAACAATAGTTCCTCAAC	
NADH-GOGAT	AtNADH F	AGTTGGGAGAAGGATGAAACCGGGAGG	Konishi et al., 2014 [133]
	AtNADH R	GTGATAGTGTTGTGTTTCATCTGGTTAAGG	
GLN1	AtGLN1 F	CATCAACCTTAACCTCTCAGACTCCACT	Ishiyama et al., 2004 [134]
	AtGLN1 R	ACTTCAGCTGCAACATCAGGGTTGCTA	
GLN2	AtGLN2 F	TTCTCCAACATGTCAGATGAGAGTGCCT	Ishiyama et al., 2004 [134]
	AtGLN2 R	CCAGGTGCTTGACCGGTACTCGAACCA	
VSP1	AtVSP1 F	CCCGGAGACCTTGCATCTA	Nicola Capstaff
	AtVSP1 R	ACACCACTTGCGTCAACTTC	
VSP2	AtVSP2 F	ACTCCAAAACCGTGTGCAAA	Nicola Capstaff
	AtVSP2 R	GTAAAGATGCAAGGCCTCCG	
TIP1.1	AtTIP1.1 F	GTGGAATCGCTGGACTCATC	Ma et al., 2004 [135]
	AtTIP1.1 R	TGATTCGAAATTACACAAACGG	
TIP1.2	AtTIP1.2 F	AAGCTGGACGTGGACCAAC	Ma et al., 2004 [135]
	AtTIP1.2 R	GCCAGAAACCCATTACGATG	
TIP3.1	AtTIP3.1 F	CCCACCGAACCACCTACC	Mao and Sun, 2015 [136]
	AtTIP3.1 R	GAACAACGAACAAAAGCA	
TIP3.2	AtTIP3.2 F	ACCACAGTACCCACCAAC	Mao and Sun, 2015 [136]
	AtTIP3.2 R	ACATAGGAAATGGCAGGA	
CYP5	AtCYP F	CTTCAGAGCTTTGTGCACAGG	Mao and Sun, 2015 [136]
	AtCYP R	AAGCTGGGAATGATTCGATG	
ACT7	AtACT F	GGTCGTACAACCGGTATTGT	Mao and Sun, 2015 [136]
	AtACT R	GAAGAGCATACCCCTCGTA	

2.1.6 Candidate gene primer design for forages

After qRT-PCR in *A. thaliana*, potential N status marker gene homologs were identified in a range of temperate legumes and monocots, using blastn, blastp [137-140] and PLAZA 3.0 Dicots and Monocots [141]. If published literature had already used these genes in *M. sativa* or *L. perenne* then these were used, otherwise they were designed in this study. Primers were needed that would cover the largest portion of sequence conserved in all species, and thus hopefully successful in the temperate forage crops used in the UK.

Multiple sequence alignments (MSA) were performed using MAFFT v7 [142] for grasses and legumes separately. The E-INS-I iterative refinement method was used as the MSA included < 50 datasets. Phylogenies were built using Newick format in iTOL v3.4.3 [143]. A consensus cds sequence was added with MSAViewer [144] and from this the most conserved sequence was used for primer design. Again these primers were designed in Primer3 [125; 126] and checked in OligoCalc [127]. Annealing of primers were checked in silico [145]. Primers based on the legume and grass consensus can be found in Table 2.2 and 2.3 respectively.

Table 2.2: Primers used in study for *Medicago sativa* testing based on legume consensus.

Gene name	Primer name	Sequence (5′ – 3′)	Source
CLC	LCCLC F	GAGGTTGCAAGTTGGTGGAG	Nicola Capstaff
	LCCLC R	TCCCAAGACACCACCAATGA	
NIR	LCNIR F	TGGCTTGCCCACAAAATTGG	Nicola Capstaff
	LCNIR R	ACTTGATGCATGGGTCTCCG	
RBCS	LCRBCS F	CCCTCTTGGGCATGCAGTAT	Nicola Capstaff
	LCRBCS R	GCCATGAATGCACTGGTTCG	
NADH-GOGAT	LCNADH F	CACTTCCGACGAAAGACCCA	Nicola Capstaff
	LCNADH R	TGGCTACACGACCCTTGTTC	
GLN	LCGLN F	CGGCCAAATGCCTTATCAGC	Nicola Capstaff
	LCGLN R	AGGATCCATTCAGGAGGGGT	
VSP	LCVSP F	TGCCCTTGTTCACCCATTCC	Nicola Capstaff
	LCVSP R	ACGTGAGTGCCTGGAAAACT	
TIP1	LCTIP1 F	CGGAGATGTTAGCACCAACG	Nicola Capstaff
	LCTIP1 R	CACCCAGACACCTTGAAAGC	
CYP	LCCYP F	CTTGGGAGTGCCACTTTGTG	Nicola Capstaff
	LCCYP R	ATGGCAATGGAATGGGTGGA	
ACT	LCACT F	ATCCCTCACAATTTCCCGCT	Nicola Capstaff
	LCACT R	AAGCTCAGTCCAAGAGGGGT	

Table 2.3: Primers used in study for *Lolium perenne* testing based on grass consensus or published primers.

Gene name	Primer name	Sequence (5' – 3')	Source
CLC	GCCLC F	GTCGCCAGGTCGTTGTATTC	Nicola Capstaff
	GCCLC R	GTGCTCCGCCTCTATAACCT	
NIR	GCNIR F	CACATCATGCGGGTCTTCTG	Nicola Capstaff
	GCNIR R	CCGAGGAAATGGAACGTGTG	
RBCS	GCRBCS F	TTCTTGACCTCCTCCAGCTC	Nicola Capstaff
	GCRBCS R	CGAGGGCATCAAGAAGTTCG	
NADH-GOGAT	GCNADH F	ATTCACACGGCGTTGAACAA	Nicola Capstaff
	GCNADH R	GTACCTCGACCACCACTTCA	
GLN	GCGLN F	TGGACTCGGTGCTGTAGTTT	Nicola Capstaff
	GCGLN R	GGCATCAACATCAGTGGCAT	
VSP	GCVSP F	GACCAGCTTGTTGTACAGCC	Nicola Capstaff
	GCVSP R	GTTGGCCACTACATGCTCG	
TIP1.1	GCTIP1 F	GCGGCAACATCAGCCTCCTCA	Nord-Larsen et al., 2009 [146]
	GCTIP1 R	TCATGACGATCTCGAACACC	
GADPH	LpGADPH F	CAGGACTGGAGAGGTGG	Petersen et al., 2004 [147]
	LpGADPH R	TTCACTCGTTGTCGTACC	
ACT	LpACT F	GAGAAGATGACCCARATC	Petersen et al., 2004 [147]
	LpACT R	CACTTCATGATGGAGTTG	

2.1.7 Forage crop growth conditions

The forage crops to be tested with designed primers were *Lolium perenne* cv. AberMagic and *Medicago sativa* cv. Daisy, found in Tables 2.4 and 2.5 below. As with *A. thaliana* above in 2.1.1, seeds for *L. perenne* were sterilised, plated and vernalised.

Seeds for *M. sativa* were sterilised as follows. Seeds were scarified prior to sterilisation with concentrated H_2SO_4 followed by six dH_2O washes. Seeds were then sterilised with a sodium hypochlorite 10 % (v/v), Triton X-100 0.05 % (v/v) (X100) solution followed by six dH_2O washes. The final wash included Nystatin 5 µg/mL (N6261), Amoxicillin 50 µg/mL (A8523), and was filter sterilised to reduce any fungal or bacterial contamination. The seeds were imbibed in this solution on a slow shake (30 ± 1 rpm) for 2 h at 4 °C, then the wash was replaced for a repeat imbibing period. Finally, seeds were washed in dH_2O and plated on the same media as above N treatments.

For cv. Daisy only, plates were inoculated with *Sinorhizobium meliloti* 1021 and *Sinorhizobium medicae* WSM419, provided by Anne Edwards (Metabolic Biology, John Innes Centre), at 7-10 days post germination to encourage nodulation. Both Sinorhizobium inoculums were prepared from single colony growth in 200 mL TY buffer (5 g Tryptone (T7293), 3 g Yeast Extract (Y1625), 1.325 g $CaCl_2.2H_2O$ (223506), in 1 L of dH2O) at 28 °C for 48 hours in orbital shaker. Cultures were centrifuged for 10 minutes at 5,000 rpm at 4 °C and diluted in FP liquid media to an optical density (OD) of ~ 0.02 at 600 nm using DeNovix® DS-11-FX+. FP liquid media was prepared using 2.5 mL $CaCl_2.2H_2O$ 272.18 mM, 3 mL $MgSO_4.7H_2O$ 162.28 mM (M1880), 3.33 mL KH_2PO_4 220.45 mM (P9791), 3.33 mL $Na_2HPO_4.12H_2O$ 125.5 mM (71649), 2 mL $FeC_6H_5O_7$ 10.21 mM (F3388), 1 mL Gibson's Trace (2.86 g H_3BO_3 (B6768), 2.03 g $MnSO_4.H_2O$ (M7634), 220 mg $ZnSO_4.7H_2O$ (Z0251), 80 mg $CuSO_4.5H_2O$ (C8027), 80 mg H_2MoO_4 (232084)), in 1 L dH_2O and pH to 6.3 – 6.7.

2.1.8 Candidate gene testing for forages with RT-PCR

RNA from plants grown on 30 mM KNO_3 plates was extracted and cDNA synthesised as in 2.1.2 and 2.1.3. NTC and NRT controls were again carried out.

RT-PCR was used to check primer suitability. Reactions in 40 – 50 µL were performed with GoTaq G2 DNA Polymerase (Promega UK Ltd. M7845) as follows; 0.75 - 1 µL cDNA, 1 µL dNTPs (10 mM), 8 - 10 µL GoTaq Green 5 x buffer, 0.25 - 0.4 µL Forward and Reverse Primer 10 µM and 0.25 µL GoTaq Polymerase. Primers used can be found in Tables 2.2 and 2.3. Reactions were performed in a G-Storm GS1 thermocycler (Gene Technologies Ltd., UK) using the following programme: 30 s at 98 °C, followed by 40 cycles of 15 s at 98 °C, 30 s at 58 °C, 20 s at 72 °C, and a final extension of 10 min at 72 °C with holding at 4 °C.

2.1.9 Gel electrophoresis and sequencing

Gel electrophoresis was used to determine expected size DNA fragment bands based on primer design. Reactions were ran using 1.5 % agarose gel (Melford Laboratories Ltd., UK, A20080) with 1 % ethidium bromide with standard laboratory procedure. Correct size bands were excised under U.V. and extracted using QIAquick Gel Extraction Kit (QIAGEN Ltd., UK, 28704) following the manufacturer's instructions with a final volume of 70 µL. PCR products were sent for sequencing using LightRun GATCbiotech service (Eurofins Genomics, UK); reactions were 5 µL PCR product, 2.5 µL Forward primer 10 µM and 12.5 µL dH_2O. Primers were redesigned to different ranges of consensus sequences based on sequencing data or failure to PCR bands of expected size. Gene primers which were successful in this procedure were tested for their primer efficiencies.

Table 2.4: Forage grass species used in this study.

Species name	Cultivar	Code/origin	Source
Lolium perenne	AberMagic	ABY-S562-2016 (Bred at IBERS)	Ianto Thomas, IBERS
Lolium multiflorum	Davinci	ABY-Bb 2593 (Obtained by IBERS)	Danny Thorogood, IBERS
Festuca arundinacea	Kora	DLF Forage Seeds	Northern Crop Dries Ltd., UK
Festuca pratensis	AberPaddock	ABY-S603-2008 (Bred at IBERS)	Danny Thorogood, IBERS
L. mulitflorum x F. arundinacea	Hykor	DLF Forage Seeds, DK	Blankney Estates Ltd., UK
L. mulitflorum x F. arundinacea	Lofa	DLF Forage Seeds, DK	Northern Crop Driers Ltd., UK
L. mulitflorum x F. pratensis		ABY-bBF 67-1986 (Line ex E.J. Lewis 1986, IBERS)	Danny Thorogood, IBERS
L. perenne x F. pratensis	Matrix	ABY-bAF 25 (Obtained by IBERS from Cates Grain Ltd., NZ)	Danny Thorogood, IBERS
Anthoxanthum aristatum		ABY-Bs 3150-2001 (Collected Spain/Portugal 1963, IBERS)	Danny Thorogood, IBERS
Phleum pratense		ABY-Bd 3342 (Collected Taiwan/Japan 2017, IBERS)	Danny Thorogood, IBERS

Table 2.5: Forage legume species used in this study.

Species name	Cultivar	Code/origin	Source
Medicago sativa	Daisy	DLF Forage Seeds, DK	Dengie Crops Ltd., UK
Medicago sativa	Luzelle	Oliver Seeds Ltd. (Bred by INRA/Agri-Obtentions, FR, 1993)	Fox Feeds Ltd., UK
Medicago sativa	Gea	DLF Forage Seeds, DK	A Pouchers and Sons Ltd., UK
Lotus corniculatus		ABY-Al 592-2013 (Breeder's Line Highgrove x Lotar, IBERS)	Danny Thorogood, IBERS
Onobrychis sativa	Aberystwyth Sanfoin	ABY-Am 361 (Bred at IBERS)	Danny Thorogood, IBERS
Trifolium pratense	AberRuby	ABY-S543-2005 (Bred at IBERS from Sabtoron (S310))	Danny Thorogood, IBERS
Trifolium repens	AberConcord	ABY-S505-1997 (Bred at IBERS)	Danny Thorogood, IBERS

2.1.10 Primer efficiency testing

Marker gene primers in Tables 2.2 and 2.3 were tested in *L. perenne* and *M. sativa* for their primer efficiencies using SYBR® Green JumpStart™ Taq ReadyMix™ (S4438) with either concentrated cDNA or 1:10, 1:100 or 1:1000 dilutions in quadruplicate; 10 µL 2 x SYBR® ReadyMix™; 5 µL cDNA, 3 µL dH_2O, 1/1 µL F/R primers 5 µM. qPCR run as above. Efficiencies were calculated for each marker gene primer set by taking an average of the individual cDNA quadruplets and plotting against the common logarithm of cDNA concentration. The linear regression of the slope was calculated, and the slope gradient value was used in the following equation to determine the % efficiency:

$$\% = \left(10^{1/slope\ gradient\ value}\right) \div 2 \times 100$$

Efficiencies that were between 90 – 110 % were designated fit to use in future gene expression assays, but those that failed were redesigned and tested as in 2.1.6 for a slightly shifted portion of consensus sequence. Those with efficiency pass scores were used in a larger forage crop screen. Primers to test across all forages can be found in Tables 2.6 and 2.7 below.

Table 2.6: Primers used in study for legumes based on *Medicago sativa* testing.

Gene name	Primer name	Sequence (5' – 3')	Source
CLC	MsCLC F	GGTTATTAGTTTCCGCGGCA	Nicola Capstaff
	MsCLC R	CTCAGCCAACTCAACCCAAC	
NIR	MsNIR F	TAGTTCGCCGGTTCCGTATT	Nicola Capstaff
	MsNIR R	TGCCAGAGAAGAAGCTAGAGAG	
RBCS	MsRBCS F	GGACATGCAAGATGGAACTCC	Nicola Capstaff
	MsRBCS R	AGGACAGCAGAGACTCGAAC	
NADH-GOGAT	MsNADH F	GTCGCCAAATTGCCTCTACA	Nicola Capstaff
	MsNADH R	TGCAGTCCACCAACATCTAGT	
GLN	MsGLN F	TGTCTTTCTGCAACAAGGTGT	Nicola Capstaff
	MsGLN R	CCCACCAACAAGAGACATGC	
VSP	MsVSP F	GAAACACAAAGCCAAAACCACA	Nicola Capstaff
	MsVSP R	ACCTTCTTCCATTACCATTTCCA	
TIP1	MsTIP1 F	CGGAGATGTTAGCACCAACG	Nicola Capstaff
	MsTIP1 R	CACCCAGACACCTTGAAAGC	
CYP20	MsCYP F	GCTACCTTTGTAATGAAGAGGCT	Nicola Capstaff
	MsCYP R	TGCAGGTCGTATTGTAATAGGTC	
ACT2	MsACT F	CGCCGGAATCCAACACAATA	Nicola Capstaff
	MsACT R	GAGGCTCCACTTAACCCAAAG	

Table 2.7: Primers used in study for grasses based on *Lolium perenne* testing.

Gene name	Primer name	Sequence (5' – 3')	Source
CLC	LpCLC F	AGTTCTGGGTGCCGTACTAC	Nicola Capstaff
	LpCLC R	ACAGAAGAGAAGACGAGGGC	
NIR	LpNIR F	GTTTTGCCGTCCTTCTCCG	Nicola Capstaff
	LpNIR R	CCGAGGAAATGGAACGTGTG	
RBCS	LpRBCS F	CAGTACCTGCCGTCGTAGTA	Nicola Capstaff
	LpRBCS R	CGAGGGCATCAAGAAGTTCG	
NADH-GOGAT	LpNADH F	CCTCTGTTTGCGTCCGTTAG	Nicola Capstaff
	LpNADH R	GTACCTCGACCACCACTTCA	
GLN	LpGLN F	GTTGGCCCTTCTGTTGGTAT	Nicola Capstaff
	LpGLN R	TGGACTCGGTGCTGTAGTTT	
VSP	LpVSP F	AGGACCCAGATTTGAAGGAGA	Nicola Capstaff
	LpVSP R	GCCACTACATGCTCGGATTC	
TIP1.1	LpTIP1 F	GCGGCAACATCAGCCTCCTCA	Nord-Larsen et al., 2009 [146]
	LpTIP1 R	TCATGACGATCTCGAACACC	
GADPH	LpGADPH F	CAGGACTGGAGAGGTGG	Petersen et al., 2004 [147]
	LpGADPH R	TTCACTCGTTGTCGTACC	
ACT	LpACT F	GAGAAGATGACCCARATC	Petersen et al., 2004 [147]
	LpACT R	CACTTCATGATGGAGTTG	

2.1.11 Larger forage screen of developed N status marker primers

To test the viability of consensus primers a larger forage screen was used with the species above in Tables 2.4 and 2.5. Seeds were surface sterilised with EtOH and plated onto water agar (3 g Agar (AGA03, Formedium Ltd. Norfolk, UK) in 200 mL dH_2O); ~ 20 seeds for each cultivar. Seeds plates were vernalised for four days at 4 - 6 °C before being transferred upside-down to a CER with temperature controlled at 23 °C and a photoperiod of 16 h light (90 µmol m^{-2} s^{-1}) and 18 h dark. Plants were germinated for 4 days on plates before being transferred to glasshouse compost pots. Ten seedlings were transplanted to 1 L pots containing a peat mix; 600 L Levington F2 peat, 100 L 4 mm grit, 196 g Exemptor® (GB84080896A, Bayer CropScience Ltd., UK) which is a chloronicotinyl insecticide.
After 3 weeks of growth in pots, the seedlings were flash frozen in liquid N_2. RNA extraction, cDNA synthesis, and RT-PCR were carried out with samples as in 2.1.2, 2.1.3 and 2.1.8 respectively with primers from Tables 2.6 and 2.7. PCR products were tested using gel electrophoresis as above to confirm expected band sizes. Percentages of correct bands for each primer was calculated for grasses and legumes. Gel images with percentage calculations can be found in Figure A2, Appendix A.

2.1.12 Developed N status marker primers forage crop qRT-PCR

Gene expression using developed primers were tested in the glasshouse with the grass species *L. perenne* cv. AberMagic and *Festulolium* cv. Hykor and the legume *M. sativa* cv. Daisy and Luzelle. Seeds were sterilised and vernalised as in 2.1.6 and germinated on water agar plates for 3 days. Seedlings were then moved to a glasshouse with 16 h light/8 h dark, 18/15 °C, relative humidity 70 %, and lit with 600 w HPS lamps and planted in p26 seed trays of medium vermiculite and 3 mm sharp sand 50:50 mix. For *M. sativa* cultivars individual plants were inoculated with 0.5 mL of

both *Sinorhizobium meliloti* 1021 and *Sinorhizobium medicae* WSM419 at 7 - 10 days post germination as described in 2.1.7. Plants were watered for 1 week with dH_2O, then subsequently watered with 50 mL treatments of 0.6, 3, 6 mM KNO_3/$Ca(NO_3)_2$ in the case of *M. sativa* and with treatments of 0.3, 0.6, 3, 6, 10, 30 mM KNO_3/$Ca(NO_3)_2$ for grasses, as in 2.1.1.

Three weeks after treatment the top 6 cm vegetative tissue was cut from 12 plants for RNA extraction and qRT-PCR as in 2.1.2, 2.1.3, 2.1.5, with primers from Tables 2.6 and 2.7. Another 12 plants were oven dried at 50 – 65 °C overnight and biomass measured. Plants were grown for another 3 weeks with dH_2O watering. At the six weeks after treatment 24 plants were cut to the above-ground base and again dried for measured for final biomass.

2.1.13 2018 field sampling of developed N status marker primers

In early May 2018 field sampling was carried out at Blankney Estates Ltd. Fields tested were in the Northwest region of Lincolnshire, England and located within 3 km of the site office (53°06'22.2"N 0°27'48.4"W, 45 m above sea level). Site specific climate data during the last decade was mean annual max/min temperature 13.8/4.9 °C and mean annual rainfall 577 mm. Soil is sandy clay with crop drying fields (BCDs) containing the following in their residual 0 – 45 cm depth topsoil before any fertiliser application for years 2011 – 2018: ammonium 0.56 – 1.6 mg/kg; nitrate 1.72 – 6.04 mg/kg; phosphorus 12.49 – 18.18 mg/kg; potassium 107.66 – 151.38 mg/kg; magnesium 45.0 – 85.42 mg/kg; pH 7.45 – 8.28 (Blankney site office data provided by Andrew Hayden).

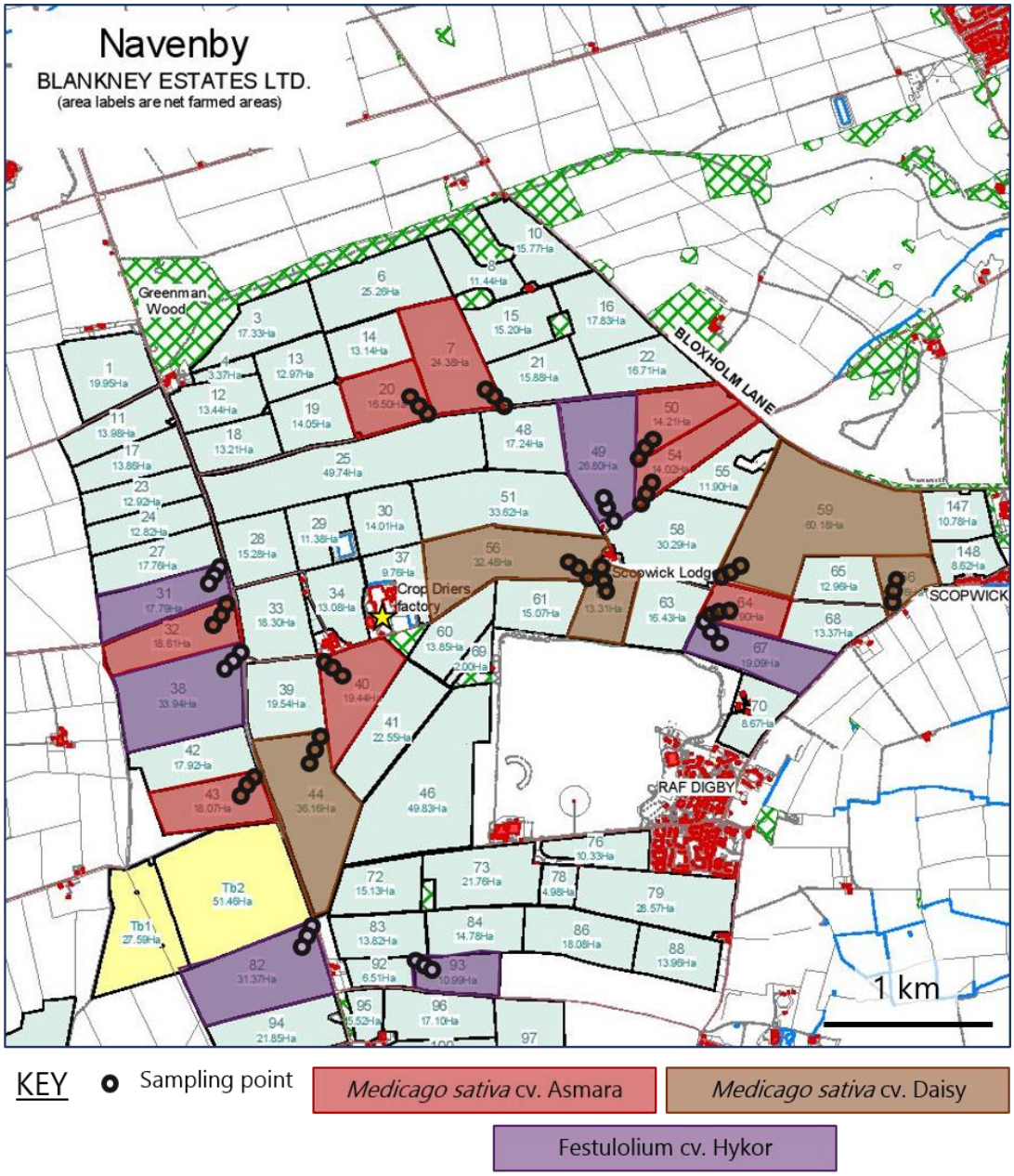
The fields sampled are indicated in Figure 2.1 below. Crops grown in fields were either *M. sativa* cv. Asmara or cv. Daisy, or *Festulolium* cv. Hykor (*Lolium perenne x Festuca pratensis*). Sample points are indicated in the figure as estimated 'dots', the first 5 m from corner of field, and the second 5 m from this to approximate centre of field, and third 5 m again from that. Samples taken were as follows:

- Soil samples – ~ 20 g of 1 - 10 cm topsoil, chosen due to practical ease of sampling and borer available, from pooled sample points to EtOH cleaned glass vials, transported at ambient temperature, stored at -20 °C within 6 hours of sampling.
- Vegetative gene expression samples - cut ~ 15 g of top 6 cm of vegetative tissue from each individual sample point, flash frozen and transported in waxed bag liquid N_2 on-site, stored at -80 °C within 6 hours of sampling.
- Vegetative tissue nutritional samples – collection of ~ 15 g fresh weight was taken at each sample point, placed in a waxed bag for chlorophyll and protein analysis at BCC Ltd., and stored at -20 °C within 2 hours of sampling.

Analysis of these samples was as follows:

- Soil samples – KCl extraction to determine nitrate concentration of topsoil, described in 2.1.14 and 2.1.15.
- Vegetative gene expression samples – RNA extraction, cDNA synthesis and qRT-PCR as in 2.1.2, 2.1.3 and 2.1.5. For qRT-PCR an average of all sample points expression was calculated, and fold changes were relative to absolute topsoil nitrate values.
- Vegetative nutritional samples – protein content analysed using Kjeldahl method as in 2.1.16, chlorophyll content analysed using Soxhlet method as in 2.1.17.

Future yield measurements for the subsequent cut of field in T ha^{-1}, ~ 6 - 21 days post sampling, were provided by Blankney Estates Ltd.



<u>Figure 2.1:</u> Fields sampled at Blankney Estates Ltd. in May 2018.

Crop grown is indicated with coloured box; red is *Medicago sativa* cv. Asmara, brown is *Medicago sativa* cv. Daisy, and purple is *Festulolium* cv. Hykor (*Lolium perenne x Festuca pratensis*). Dots indicate area where soil and vegetative samples were taken for analysis.

2.1.14 Soil extraction with KCl

Soil stored at -20 °C was defrosted to RT and sieved to 5.6 mm. A mixed sample of 10 ± 0.02 g soil was transferred to a 125 ml Wide Neck bottle (Azlon™ HDPE Wide Neck Round Bottles, Azlon™ BLH0125P) and 50 mL KCl 2.0 M extraction solution added. A blank extraction with no soil was also carried out. This was shaken on an orbital shaker at 5 Hz for 2 hours ± 10 minutes, and then filtered through a Whatman No.4 filter paper (150 mm diameter, WHA1204320) and filtrate retained. Filtrate was used to determine nitrate concentration as below in 2.1.15.

2.1.15 Spectrophotometric determination of nitrate in soil extractions

A standard nitrate solution of 50 µg/mL was prepared using KNO_3 in dH_2O. From this, solutions were prepared in 10 mL KCl 2.0 M extraction solution to contain 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 µg/mL as working standards for calibration regression.

A reduction-diazotisation reagent was prepared within a fume hood by adding the following 'solution a' to 'solution b'; 'solution a' was 400 mg vanadium chloride (208272) to 50 mL HCl 1.0 M shaken gently until dissolved; 'solution b' was 200 mg of sulphanilamide ≥ 99 % (S9251) and 10 mg NEDD ≥ 98 % (N-(1-naphthyl)ethylenediamine dihydrochloride, 33461, Supelco®) in 400 mL dH_2O.

To standard 3.5 mL cuvettes (Sarstedt Limited, 67.755) 1 mL of either working standard or soil extracted filtrate was added, then 800 µL of reduction-diazotisation reagent. After 20 hours at RT absorbance was measured at 540 nm. Working standard readings were used to fit a calibration regression, with R^2 of ≥ 0.98. The linear equation was used to determine soil extracted filtrate concentrations, with blank reading subtracted and multiplied by both the dilution factor of KCl solution (5) and the dilution factor of soil sampled (10). This provided the nitrate concentration in mg/kg of the original soil.

2.1.16 Protein analysis using Kjeldahl method

Protein analysis using the Kjeldahl method was carried out at British Chlorophyll Company Limited (Blankney Estates, Navenby). Vegetative tissue from field trials was analysed to determine N content through conversion to NH_3, and therefore calculate protein content. Frozen samples were ground at RT, and ~ 1 g measured on a Whatman No.1 filter paper (125 mm diameter, WHA1201331). This was folded into a tight parcel and reweighed. The sample parcel was added to a 500 mL round bottom Kjeldahl flask for digestion of organically bonded N into NH_4^+. A catalytic salt mix was added to aid digestion; 35 g of 50:1 sodium sulphate:cupric sulphate in each flask. In a fume cupboard 30 mL H_2SO_4 95% (GPR grade) was added, swirled gently, and an adapter placed in the flask neck. The flask was heated with a Bunsen burner, gently at first and increasing up to 350 – 380 °C. Digestion took ~ 30 – 60 mins and was deemed complete when the solution turned clear or pale blue with no brown material left. Once colour change was complete, the flask was removed from the fume cupboard to cool to RT, but not allowed to solidify.

When cooled the flask adaptor was removed for distillation and ~ 25 mL dH_2O added to ensure solution didn't set, swirling to dissolve all digested material. Anti-bumping crystals were added to each flask, and the neck of the flask was greased with petroleum jelly to ensure a seal when connecting the flask to the distillation unit. When all joints were sealed the condenser spout was placed above a receiving vessel containing a trap solution; 3 mL hydrochloric acid 1 M (Analar Grade) with a few drops of Methyl red indicator and 150 mL dH_2O in a 500 mL flat bottom round flask. Ensuring the reservoir at the top of the distillation unit was closed, 75 mL caustic soda solution 33 % was added slowly, opening the reservoir to gently add the caustic solution until there was a colour change. Once added the reservoir was closed and water ran through the condenser. The flask was gradually heated again with a Bunsen burner, firstly to the side of the flask then slowly moved to the centre of the flask once the solution began to bubble gently. Heating continued until the mixture started 'banging', then heat was removed. The condenser was disconnected to prevent any of the

heated mixture passing through the distillation unit and contaminating the trap solution, which now contained N converted to NH_3.

The trap solution was titrated with sodium hydroxide 0.1 M until the solution turned from pale pink to yellow. The burette reading prior and after titration was recorded and used to calculate protein as follows:

$$\frac{Titration\ difference\ (mL)}{Weight\ of\ ground\ sample\ (g)} \times 100 = \ Protein\ in\ sample\ \%$$

The protein content (%) could be used to calculate total dry weight % of protein in tissue using the moisture % of original tissue:

$$\frac{Protein\ in\ sample\ \%}{(100 - moisture\ \%)} \times 100 \ = \ Total\ dry\ weight\ protein\ (\%)$$

2.1.17 Chlorophyll analysis using Soxhlet extraction

Chlorophyll analysis using the Soxhlet extraction method was carried out at British Chlorophyll Company Limited (Blankney Estates, Navenby). Vegetative tissue from field trials was analysed to determine crude chlorophyll content. Ground samples from protein analysis in 2.3.10 were used, and ~ 5 g measured on a Whatman No.4 filter paper (150 mm diameter). The paper was folded, and sample parcel fitted into an extraction thimble. This was plugged with cotton wool and placed into a Soxhlet extraction apparatus. This was connected to a 250 mL flat bottom flask containing anti-bumping crystals, with weight recorded. ~ 100 mL of DCM was added to the apparatus, and water ran through the condenser, with heating from a Bunsen burner. Extraction was performed until there was no green colour in the liquid surrounding the thimble. Once extracted, the thimble was removed, and the DCM distilled from flask. Once distilled, the flask was placed in an oven set at 105 °C for 20 minutes. Flask was then removed and cooled to RT. The flask was weighed and recorded, and crude chlorophyll calculated:

$$\frac{Final\ flask\ weight\ (g) - initial\ sample\ weight\ (g)}{Initial\ flask\ weight\ (g)} \times 100 = Crude\ chlorohpyll\ \%$$

The crude chlorophyll content (%) could be used to calculate total dry weight % of chlorophyll in tissue using the moisture % of original tissue:

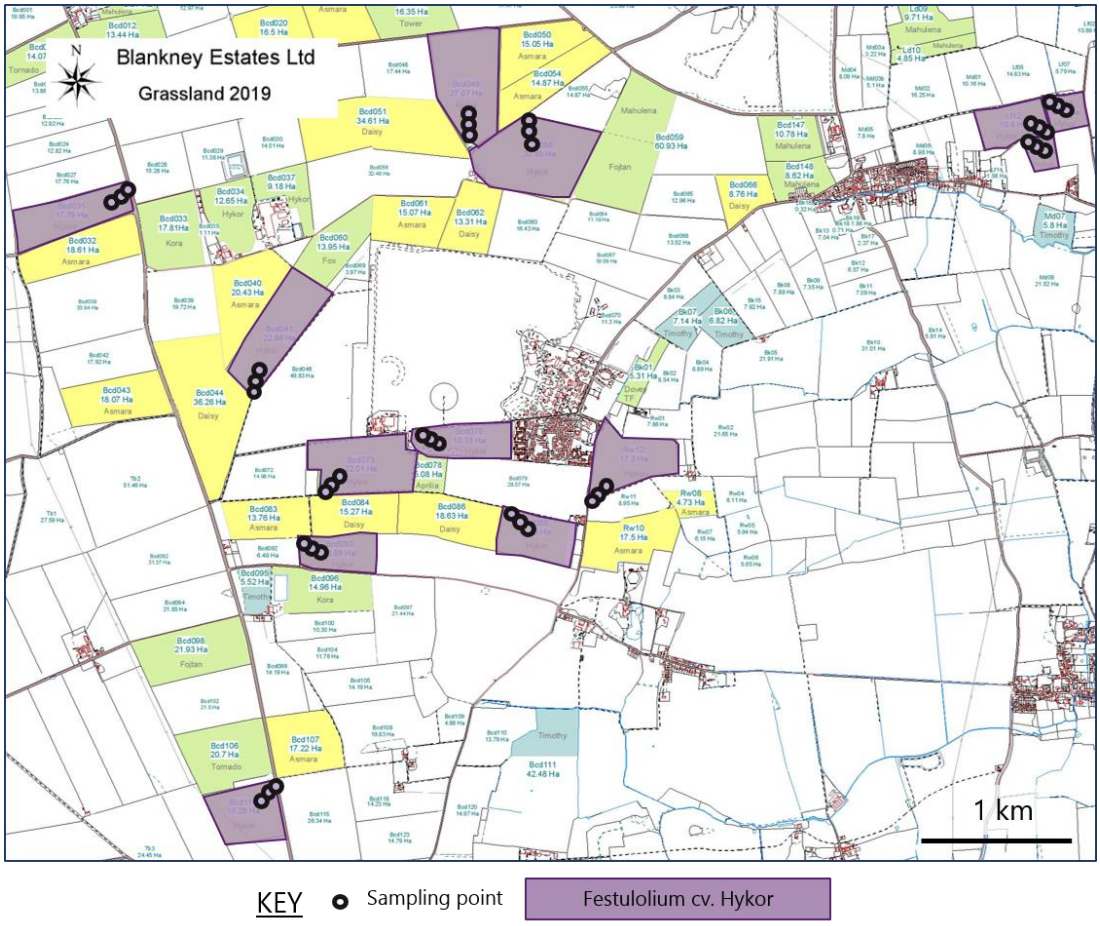
$$\frac{Crude\ chlorophyll\ in\ sample\ \%}{(100 - moisture\ \%)} \times 100 = Total\ dry\ weight\ chlorophyll\ (\%)$$

2.1.18 Further qPCR of developed N status marker primers in *Festulolium*

Following the initial testing of *Festulolium* cv. Hykor in 2.1.10, and its extensive use on collaborator sites, gene expression was tested with more NO_3^- treatments. Seeds were sterilised, vernalised and planted as above, with plants again watered for 1 week with dH_2O. Treatments were then applied with the following NO_3^- mM concentrations: 1, 5, 8, 12, 15, 17, 19, 21, 23, 26, 29, 32, 35, and 38 mM. To avoid problems with cation balancing for KNO_3 and $Ca(NO_3)_2$ in 2.1.1 with higher concentration solutions, the treatments were prepared with both nitrate sources in a 1:1 NO_3^- ratio. qRT-PCR was performed with N status marker primers for *NIR*, *NADH-GOGAT*, *RBCS* and *TIP1* as before in 2.1.12.

2.1.19 2019 field sampling of developed N status marker primers

Sampling was again carried out in 2019 at Blankney Estates Ltd. as in 2018 detailed in 2.1.13, this time only for fields cultivating *Festulolium* cv. Hykor. Figure 2.2 below shows the fields sampled. Sampling and analysis were as before.



<u>Figure 2.2:</u> Fields sampled at Blankney Estates Ltd. in April 2019.

Crop grown is indicated with coloured box; purple is *Festulolium* cv. Hykor (*Lolium perenne x Festuca pratensis*). Dots indicate area where soil and vegetative samples were taken for analysis.

2.1.20 Modelling N status marker gene expression

Expression data from glasshouse experiments was investigated to produce a potential model for field analysis. The hypothesis was that expression data could be used to assess either soil nitrate or future yield. The idea to use a Gaussian process approach to the data as well as the initial R script [148] was written by Dr Alexander Calderwood (Crop Genetics, JIC) after discussing the aims of the project with N.C.; initial script included assessing all gene expressions and investigating both soil nitrate and future yield. The script was then edited by N.C. for soil nitrate only as future yield was too variable in the field. Moreover only the genes *NADH-GOGAT*, *NIR* and *TIP1* were included as *RBCS* was also too variable for analysis. This R script for analysing 2018 and 2019 data combined can be found in Table A1, Appendix A. The whole script used common packages installr [149], tidyverse [150], cowplot [151], ggplot2 [152], and data.table [153] to build and plot data.

Simply, the R script used the normalised geomean $2^{\wedge -\Delta CT}$ of 2018 glasshouse and field *Festulolium NADH-GOGAT*, *NIR* and *TIP1* expression data from 2.1.12 and 2.1.13, to generate values for 'mean.delta.ct.norm'; values for 0.3 mM were ignored due to the extreme outlier of *TIP1* expression. Glasshouse values are plotted against soil 'NO3' shown in Figure A4, Appendix A, with all 2018 graphs given a red border. The glasshouse values were then used to build a Gaussian model using the package GauPro [154] with the known minimum and maximum values for soil NO_3^- specified. This required values for 'yhat' or the mean of all gene expression values for 'mean.delta.ct.norm', and 'xpred' as predicted soil NO_3^-, again shown for each gene in Figure A5, Appendix A. The field 'mean.delta.ct.norm' values were then fed into the model to give 'yhat' and the 'xpred' predicted soil NO_3^- calculated based on each gene, as Figure A6, Appendix A. All the 'yhat' values for individual genes were then calculated using the model all together as the series 'P(D|NO3)' and a plot created against 'predicted equivalent greenhouse NO3', provided in Figure A7, Appendix A. The plot was then compared to actual field extracted NO_3^-

from sampling to see if there was agreement in the model and the actual values, of which there was for most fields.

All of the above was repeated for 2019 glasshouse and field data from 2.1.18 and 2.1.19 respectively; all graphs can be found in Figures A8, A9, A10, and A11 Appendix A with all 2019 graphs given a blue border.

Finally the R script was run for all samples, with models built using 2018 and 2019 glasshouse data and used to assess 2018 and 2019 field data. Again this was compared to actual extracted soil NO_3^- values, with plots available in Chapter 3 and results discussed.

2.2 NITRATE AVAILABILITY MONITORING USING SOIL SENSORS

2.2.1 Nitrate-selective sensor construction

Nitrate-selective sensors were constructed executing patent for Soil Chemistry Sensors Pub. No. WO/2014/096844 [155] with the following method. A general schematic can be found in Appendix B (Figure B1).

Boxes of 1.25 mL graduated tips (TipOne® S1112-1830, STARLAB) were silanized to prevent membrane leakage by dipping the tip to a depth of ~ 1 cm with Repelcote™ (Dow Corning® Repelcote VS water repellent, a 10% emulsion of a polydimethylsiloxane fluid, silica filler and non-ionic emulsifier). These were re-racked into original box and dried overnight (o/n) in a fume hood at RT. Two membrane solutions were prepared in sterilised 2 mL glass vials cleaned with 70 % EtOH, as follows;

- Ion-selective membrane solution containing 12 mg tridodecylmethylammonium nitrate (Selectophore™ ≥ 99.0 %, 91664), 2 mg methyltriphenylphosphonium bromide (98 %, 130079), 46 mg poly(vinyl chloride) (high molecular weight, 81392), 10 mg nitrocellulose (Amersham Hybond ECL, RPN2020D, 0.45 µM, 200 x 200 mm, GE Healthcare), and 130 mg 2-nitrophenyl octyl ether (99 %, 73732) in 2 mL final volume of tetrahydrofuran solvent (EMD Millipore, ≥ 99.9 % 1081100500).
- Reference membrane solution containing 2 mg potassium tetrakis(4-chlorophenyl)borate (Selectophore™ ≥ 98.0 %, 60591), 45 mg polyethylene glycol 3500 (1546547), and 10 mg nitrocellulose in 2 mL final volume of tetrahydrofuran solvent.

Both solutions were covered with aluminium foil then capped and sealed with parafilm to avoid evaporation of solvent. Solutions were shaken at ~ 150 rpm using an orbital shaker o/n at RT to dissolve reagents thoroughly. The salinized tips were dipped into either ion-selective or reference membrane solution to a depths of ~ 2 cm and left to dry in a fume hood for 48 hours at RT. This left a membrane in the tip of ~ 2 - 3 mm.

Two backfill solutions were prepared in 200 mL Duran flasks;

- Ion-selective backfill solution containing 2.202 g potassium nitrate (ReagentPlus® ≥ 99.0 %, P8394), and 1.49 g potassium chloride (BioXtra ≥ 99.0 %, P9333) in 200 mL dH_2O.
- Reference backfill solution containing 3.12 M potassium chloride, 20 mg silver chloride (99.999 % trace metals basis, 204382), 1.8 g sodium chloride, and 0.18 g naphthol green B (Technical grade, N7257) in 200 mL dH_2O (note that the naphthol green B dye is only used to colour the solution).

One mL of the corresponding backfill solution was loaded into the top of the corresponding membraned tip. Air bubbles were displaced with gentle flicking of the tips. Backfilled tips were stored in their original rack box half-filled with 100 mM potassium nitrate at RT o/n or until future use.

Sensor wires were prepared by stripping ~ 1 cm of plastic coating from 1.5 m lengths of wire. Black and brown sensor wires were used for ion-selective and reference tips respectively (RS Components Ltd., 192-3998 and 400-0199 respectively). At one end of the sensor wire ~ 7 cm of silver wire 99.9 % 0.7 mm diameter (Palmer Metals, FS-RW070) was clamped to each sensor wire. The silver end of the sensor wires was coated in 50 mM potassium chloride and then threaded through an earplug (RS Components Ltd. 771-4894) to secure into tips on construction. Sensor wires were placed into the top of individual membraned and backfilled tips corresponding to their sensor type using a disposable needle to allow gas to escape. These were secured with black cable ties (RS Components Ltd. 233-455). Finished sensors were sealed/secured depending on future use; sensors to be used in the field would be glued with and those for columns were wrapped in 2 x 5 cm strips of parafilm 'M' 100 mm x 38 m (Slaughter Ltd., 291-0057).

2.2.2 Nitrate ion-selective sensor calibration

Pairs of each sensor type were formed into sensor sets and connected to a GP2 logger (Delta-T Devices Ltd., Cambridge, UK); within sets ion-selective sensors were (+) channels and corresponding reference sensor

were '-'. All available channel pairs were used in a logger (n=12) unless a Delta-T SM300 soil moisture and temperature sensor was also connected (which reduces channels available to 10).

A DeltaLINK 3.6.2 (Delta-T Devices Ltd.) mM programme was installed on the logger for calibrations; 'Voltage, not powered' and circuit detection and power channel disabled. Sensors were placed into solutions of 300, 30, 3 and 0.3 mM KNO_3 sequentially whilst the programme was running, for at least 5 min each. This measured the electrical potential of sensors in each concentration and recorded them in mV. Data was captured and an average across one-minute period was calculated. This was fitted in a linear regression for each sensor alongside the known $\log_{10}(x)$ NO_3^- concentration using Excel® 2016 (Microsoft®). This provided a calibration equation for each sensor set:

$$mV = (m \times \log_{10} NO_3^- \ mM) + c$$

Any sensors with a slope factor 'm' not between 46 – 64 mV were considered not viable for use. Nonviable sensors were reconstructed, usually with only a prepared backfilled tip change required. Viable sensors were stored in 100 mM KNO_3 until use.

2.2.3 Sensor running and data analysis

Sensors were placed in the soil in the field or columns with care taken to limit disturbance to tip membranes. The output voltage for each sensor set was recorded with the same programme as calibrations but at 1 - 30 minutes intervals, depending on resolution desired for the experimental set-up. During experiments, sensors which showed signs of leaking or whose signal behaved erratically were monitored. At the end of an experiment all sensors were recalibrated as in 2.2.2, and those which did not fit into the above criteria were removed from the data analysis.

The data was analysed with a laboratory temperature slope coefficient included to compensate for temperature changes derived from the Nicolsky-Eisenmann relationship [156]; experimental mV is compared to

theoretical calculated mV across a range of temperatures to formulate a linear coefficient of compensation:

$$Temperature\ compensation = (0.4045 \times °C) + 93.61$$

After a few days of settling when the sensors were monitored for stability and viability, a 'resting period' was initiated; about 1 week of programme running with only dH_2O watering of the soil every 2 - 4 days.

The arithmetic mean 'mM' value for 12 hourly periods were calculated using calibration equations and temperature compensation in Excel, at 0000 hours and 1200 hours. These were plotted against time in GraphPad Prism 7 (GraphPad Software Inc.), with soil temperature and moisture measurements plotted at 10-minute intervals. Datasets were analysed using repeated measurement ANOVA in RStudio to determine statistical significance between columns at different depths across time.

Although all columns should have the same starting residual nitrate in soil before the experiment began as they came from the same field site, if there was a significant difference then the delta mM (Δ mM) could be calculated; the mean value for the 0 - 6 day 'resting period' could be subtracted from both 'resting period' values and subsequent 12 hourly 'running period' values. Furthermore, the dH_2O watering 'resting period' helped to negate any differences between columns.

2.2.4 Column experiments of *Lolium perenne* monocropping

Silty clay soil was sourced (Church Farm, John Innes Centre, 52°37'59.8836" N 1°10'46.3440"E) and placed into 4 plastic opaque columns (height = 50 cm, inside diameter = 15.4 cm) with 5 drainage holes at base; columns were made by John Humble, John Innes Centre Workshop. Holes for sensors we made at three levels, top (1 cm depth), middle (25 cm depth) and bottom (49 cm depth). Mini suction lysimeters (10 Rhizon SMS, Rhizosphere Research Products B.V., Wageningen, The Netherlands) could also be used to collect soil water samples from the base drainage holes, for conventional soil water analysis. A schematic and

photograph of the column setup can be found in Chapter 4 as Figure 4.2 and Appendix B as Figure B2 respectively. Columns were placed in a glasshouse with 16 h light/8 h dark, 18/15 °C, relative humidity 70 %, and lit with 600 w HPS lamps.

Water holding capacity of each soil column was determined and watered with dH_2O every 2 - 4 days to a similar water capacity to allow for nutrient movement through a soil profile. One to three NO_3^- soil sensors were placed at each of the three levels in all four columns and recorded NO_3^- concentrations as described above in 2.2.3.

At the end of the 'resting period' (6 - 12 days) the experiment began with watering of either H_2O or KNO_3 and planting of *L. perenne* cv. AberMagic seedlings in 3 of 4 columns. An experimental design table is found in Chapter 4 as Table 4.1. KNO_3 treatments were equivalent to 57 kg ha^{-1} as the standard in UK forage agriculture [157], which is 10.76 g KNO_3 in 1 L for each column. Seeds were surface sterilised with EtOH 70 % (v/v) and then germinated in a 10 cm round petri dish for 6 days before transplanting into columns at a seeding rate of 43.68 kg ha^{-1}, ~ 400 seedlings per column factoring in germination rate.

Columns were watered every 2 - 4 days. After 4 weeks all the plants were cut to their vegetative base to simulate cropping in one column, and the tissue was oven dried overnight and biomass recorded. At 8 weeks all three planted columns were cut to the base and again the tissue was dried overnight, and the biomass recorded. The whole experiment was repeated four times.

For one experiment the soil water was collected from the base drainage holes. Water was collected every 1 – 4 days. This was analysed with the spectrophotometric method described in 2.1.15 for soil extractions, but with 1 µL of sample.

2.2.5 Column experiments of *Lolium perenne* and *Medicago sativa* intercropping

Column experiments were repeated with *L. perenne* and *M. sativa* intercropping. Four opaque columns were again used, with the

experimental design for the set-up found in Chapter 4 as Table 4.2. Nitrate treatment was as before, this time for only 2 of the 4 columns. Seeds were sterilised as above, with a seeding rate of 80:20 grass:legume; 0.66 g of *L. perenne* cv. AberMagic and 0.08 g *M. sativa* cv. Daisy to 3 columns. This planting density simulated that used by the UK forage industry.

After 4 weeks, the plants were cut to the base in two columns and the biomass measured. At 8 weeks, the plants were cut in all 3 columns and the biomass measured. The whole experiment was repeated twice, due to time constraints of project.

For one experiment, the soil water was again collected for conventional soil water analysis from the base drainage holes. Again, as with monocrop columns, this was taken every 1 – 4 days, and stored for analysis at -20 °C. Testing was performed as in 2.1.15, using a 1 µL of sample.

2.3 FULVIC ACID TESTING OF FORAGE CROPS

2.3.1 Fulvic acid materials

Two fulvic acid materials (FAs) were acquired, VitaLink Fulvic (sourced from Holland Hydroponics & Horticulture, UK [158]) and MPXA (F.A.R.M. Co., California, USA [159]). These were called VFA and MFA for all subsequent work. Stock solutions were used for chemical analyses, and diluted solutions based on company guidelines were used in plant applications. VFA stock solution was the solution as purchased, with a 1 % dilution in dH_2O used in applications, with pH 6.0. An MFA stock was made with ~ 1 g MPXA in 1 L dH_2O and a 0.5 % dilution of this was used in applications, with pH 4.8. A year in industry student, Freddie Morrison (F.M.) performed some of the experiments with my supervision and guidance from other experts as indicated.

2.3.2 Initial test of FAs on forage crop vegetative biomass

An initial screen of 17 species of forage grasses and legumes was carried out with autoclaved treatments of either dH_2O, 1 % VFA or 0.5 % MFA. Information of species tested can be found in the Tables 2.4 and 2.5.

Seeds of all cultivars were sterilised, plated and vernalised as in 2.1.7, with ~ 200 seeds for grasses and ~ 100 seeds for legumes. Seedlings were grown for six days. Similar sized seedlings were transferred to 1 L pots filled with Church Farm soil (see 2.2.4) in triplicate; there were 40 grass seedlings established per pot (apart from *Phleum pratense* where 60 seedlings were used) or 20 legume seedlings per pot (apart from *Onobrychis sativa* and *Medicago sativa* cv. Gea where 10 seedlings were used due to poorer germination rate). These planting densities simulated those used by the UK forage industry. The day after transfer to soil, pots were treated with 10 mL if either dH_2O, VFA or MFA. This liquid application was made evenly over the soil surface. Seedlings were grown in glasshouse

conditions for 21 days, with watering every 3 – 5 days. Any weeds growing in pots were recorded on day 7, 12, and 17 and designated as a 'grassy weed' (e.g. couch grass), or a 'leafy weed' (e.g. buttercup, nettle, chickweed).

At 21 days post-treatment photos were taken for all species apart from *L. mulitflorum* x *F. arundinacea* cv. Lofa and *O. sativa* were seedling establishment was too low. Photos were taken using an iPhone SE with iOS 12 and imported as JPGs into Powerpoint for figures. Plants were cut to the base of plants and the tissue collected and dried in oven at 55 – 60 °C overnight. Biomass was recorded, and the percentage yield difference of FAs compared to NA were calculated in Excel. Soil samples of 10 ± 0.1 g treatment pots were taken for three grasses and three legumes and freeze dried (VirTis, Benchtop SLC model 4KBTZL-150, 2022131) for pH analysis in 2.3.3. The whole initial test of FAs with UK forage crops was carried out twice.

2.3.3 Soil pH measurement

Freeze dried soil samples were sieved to 2 mm into glass Duran flasks containing 50 mL dH_2O and placed on an orbital shaker at RT for 60 ± 5 mins at 5 Hz. Samples settled for 60 mins and the pH was recorded.

2.3.4 FA Chemical analysis standard solutions

Chemical analysis of FAs required standard solution and were made in conjunction with F.M. To determine the soluble dry weight of each FA they were washed in MeOH 100 % (v/v) and the soluble layer evaporated in low boiling point mode at 60 °C and no heat lamp (Genevac EZ-2 Elite). VFA yielded 0.579 mg/mL and MFA 1.521 mg/mL. VFA is a brown, slightly sticky powder, and MFA a very dark brown sticky gel. Standard concentrations of 0.01, 0.05 and 1 g/mL in dH_2O was made for each for analysis.

2.3.5 FA Inductively Coupled Plasma Spectrometry

Elemental analysis of 0.05 g/mL for total N, C and trace elements using Inductively coupled plasma-optical emission spectrometry (ICP-OES) and Inductively coupled plasma mass spectrometry (ICP-MS) for VFA and MFA standard solutions. These were performed offsite for VFA at Computational and Analytical Sciences, Rothamsted Research, Harpenden and for MFA at Biological Services, UEA, Norwich. Tables 2.8 and 2.9 show the results of this analysis, calculated as mg/L. Some detected contents were deemed negligible when accounting for elemental concentrations in growth media or soil and could be discounted as having an impact on plant growth; for example, a concentration of titanium at 0.0618 mg/L in MFA 0.5 % solution is not likely to affect plant yields. This data, along with any compounds found with Mass Spectral data below was used to formulate nutritional control with similar elemental contents and pH as both FA sources.

<u>Table 2.8:</u> VFA detectable content in mg/L for individual elements by ICP-OES and ICP-MS if 0.5 % solution.

Al 396.153	Ca 315.887	Co 59	Cu 63	Fe 238.204	K 766.490	Mg 279.077
$4.33e^{-1}$	19.06	$2.55e^{-3}$	$7.49e^{-3}$	$4.20e^{-1}$	1.72	8.90
Mn 55	Ni 60	S 181.975	Zn 66	Na 589.592	Total N	Total C
$1.07e^{-1}$	$5.95e^{-3}$	37.11	$3.44e^{-2}$	15.90	1.51	0.03

<u>Table 2.9:</u> MFA detectable content in mg/L for individual elements by ICP-OES and ICP-MS for 0.5 % solution.

Ti 337.280	Mo 202.032	Cr 267.716	Cd 226.502	P 213.618	Zn 213.857
$6.18e^{-2}$	$3.57e^{-4}$	$4.86e^{-4}$	$1.25e^{-4}$	$4.24e^{-2}$	$4.43e^{-3}$
Co 228.615	Ni 231.604	Cu 324.754	Fe 259.940	Mn 259.372	Total N
$1.06e^{-4}$	$2.54e^{-4}$	$1.13e^{-3}$	2.53	$1.13e^{-3}$	8.59

2.3.6 FA Thin Layer Chromatography

FA standards of 0.01 g/mL were analysed by F.M. under the guidance of Dr Michael Stephenson (Metabolic Biology, John Innes Centre) using Thin Layer Chromatography (TLC) on TLC Silica gel 60 plates (HS3822 00 00, Merck Millipore Ltd., Watford, UK). Standard solutions were dissolved in the solvents Hexane:Ethylacetate in 2:1, 1:1, 1:2, 1:3, and 100 µL applied to the plate. Plates were examined under short and long wave UV and subsequently stained with vanillin stain: EtOH 95 % (v/v), sulphuric acid 5% (v/v) and vanillic aldehyde 40 mM (W310700). No movement was found from base line following staining, suggesting FA compounds were not reactive to UV.

Increased standard concentration was tested, 1 g/mL, with solvents mixes of Dichloromethane:MeOH and Ethylacetate:MeOH in 1:1, 1:2 and 1:3 mixes were tried, as well as pure MeOH. Only a very faint 'smudge' was found, suggesting the compounds for both VFA and MFA were extremely polar.

2.3.7 FA Liquid Chromatography Mass Spectrometry

Standard sample of 0.01 g/mL were analysed for their components by Liquid Chromatography Mass Spectrometry (LC-MS IT-TOF 010024700170, Shimadzu) by F.M. under the guidance of Dr Lionel Hill (Metabolic Biology, John Innes Centre). Run information was as follows:

- Column: C18 chain column (Kinetex® 2.6 µm EVO C18 100 Å, LC Column 100 x 2.1 mm).
- Elution: Gradient of acetonitrile (solvent B) versus 0.1 % formic acid in HPLC water (solvent A), run at 0.5 mL min^{-1} and RT with 3 % B each time point: 0 min; 1 min; 6 min; 6.5 min; 6.6 min; 9 min hold with detection below.
- Detection and data acquisition: Positive polarity ionization with detection with 6.25 Hz; Curved Desolvation Line (CDL) temperature at 250 °C; Heat block temperature at 300 °C; nebulizing gas flow 1.5 L/min. Detections were monitored at 200 – 600 nm for

UV/visible absorbance with positive mode electrospray mass spectrometry of 200 - 2000 m/z full spectra; Tandem mass spectrometry of precursor ions at an isolation width of 3 m/z; Collision-induced dissociation (CID) 50%, and Ion Energy 50%.

In VFA a peak cluster identified as polyethylene glycol (PEG) chains of different lengths was detected, a with a charge envelope from 0.1-1 m/z and repeating ion mass units of 44 Da. LC-MS was repeated with samples, with a standard of 0.01 g/mL PEG 400 (202398). Peak in VFA was confirmed with this standard for its concentration using NIST Atomic Spectra database (v14, National Institute of Standards and Technology, Maryland, USA) [27].

Samples were also run in reverse phase using High Performance Liquid Chromatography (HPLC Dionex Ultimate 3000) with both a Charged Aerosol Detector (CAD Dionex Corona Veo RS). Run information was as follows:

- Column: Hydrophilic Interaction Chromatography (HILIC) column (Luna® 5 µm HILIC 200 Å, LC Column 250 x 4.6 mm).
- Elution: Linear low of acetonitrile (solvent B) versus 0.1 % formic acid in HPLC water (solvent A), run at 10 mL min^{-1} and RT with 10 % B each time point increasing every 5 mins to 95 % from 10 – 400 mins; hold at 95 % for detection for 5 mins; decrease to 60 % for detection for 2 mins; increase to 100 % until competition.
- Detection: Partial injection mode with fluorescence detection at wavelengths 220, 250 and 280 nm; fractions pooled and evaporated at 40 °C with CAD peak width limited to 0.1.

In VFA, peak cluster suspected as PEG was again detected, but poor range in chromatograms suggested other compounds were extremely polar as found by TLC.

2.3.8 FA Gas Chromatography Mass Spectrometry

Standard samples of 0.01 g/mL were analysed for their components by Gas Chromatography Mass Spectrometry (Agilent GC-MS Single Quad Mass Spectrometer (7890/5977), Agilent technologies, California, USA) by

F.M. under the guidance of Dr Paul Brett (Metabolic Biology, John Innes Centre). Run information was as follows:

- Column: Zebron ZB5-HT INFERNO column (7HG-G015-02-GGA; length 30 m with 5 m guard, internal diameter 0.25 mm, film 0.1 µm; Phenomenex).
- Carrier gas: Helium at constant flow of 1.1 mL/min.
- Inlet temperature: 250 °C
- Injection volume: 1 µL
- Injection mode: split (10:1)
- Oven temperature: Initial temperature 60 °C
  Ramp 10 °C/min to 300 °C
  Hold: 300 °C for 5 min
  Equilibration time: 0.5 min
- Auxiliary temperature: 290 °C
- Acquisition mode: SCAN between 50 - 600 m/z
- Sample preparation: Derivatised with MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide, 394866) instead of dH_2O solution.

Data was acquired with Agilent Masshunter Qualitative Analysis (B.07.00) and peaks were identified by METLIN [160; 161]. In VFA a PEG cluster was again found, as in above LC-MS, (see Figure C3, Appendix C) In MFA citric acid derivatives synonymous to isomers including R-(homo)2-citrate were detected (see Figure C2, Appendix C), likely in a complex with $Fe^{(2+)}$ which is in high levels comparable to other elements (see Table 2.9). GC-MS was repeated with samples, with standards 0.001 g/mL citric acid (C1909) for MFA, and 0.01 g/mL PEG 400 (202398) for VFA. Peaks in MFA and VFA was confirmed with these standards as above with NIST Atomic Spectra database (v14, National Institute of Standards and Technology, Maryland, USA) [162] (see Figures C2 and C3, Appendix C).

2.3.9 FA control solutions

Controls for VFA and MFA were formulated using the chemical data above in Tables 2.8 and 2.9, and with the above LC-MS and GC-MS analysis (2.3.7 and 2.3.8). Compounds which could impact plant growth when compared to media or soil contents, even in tiny amounts, were accounted for. These were named VC and MC accordingly and can be found in Tables 2.10 and 2.11. All solutions were autoclaved prior to assays.

Table 2.10: VFA control solution (VC) components for 1 % application as in 2.3.1.

Compound is given, with Sigma-Aldrich catalogue number, and mg/L required.

KNO_3	Sucrose	$AlCl_3$	$CaSO_4.2H_2O$	$FeSO_4.7H_2O$	K_2SO_4
P8291	84097	563919	C3771	F8263	P0772
1.95	125.5	7.75	163.7	4.2	13.2
$MgSO_4$	$MnSO_4.xH_2O$	$Zn(NO_3)_2.H_2O$	NaCl	Na_2SO_4	PEG-400
M7506	229784	230006	S7653	S6547	202938
88.15	0.65	0.15	6.4	74.4	62.75

pH to 6.0

Table 2.11: MFA control solution (MC) components for 0.5 % application as in 2.3.1.

Compound is given, with Sigma-Aldrich catalogue number, and mg/L required.

NH_4NO_3	$CuSO_4 \cdot 5H_2O$	$FeSO_4.7H_2O$	Monosodium citrate
A7455	C8027	F8263	71498
24.5	8	12.45	45.6

pH to 4.8

2.3.10 FA Nuclear Magnetic Resonance

Analysis above was used to produce elemental controls for FAs to use in plant and microbial assays, and Nuclear Magnetic Resonance (NMR) was carried out to elucidate which carbon compound types or ratios are present in the extractions. NMR and analyses of VFA and MFA were carried out by Dr Juan Carlos Muñoz-Garcia with supervision from Prof Yaroslav Khimyak (both School of Pharmacy, University of East Anglia).

^{1}H-decoupled ^{1}H-^{13}C cross polarization (CP) and CP single pulse (CPSP) solid state NMR experiments were performed at 20 °C using a 7.05 T Bruker Avance III spectrometer equipped with a 4 mm triple resonance probe (Bruker©, Fällanden, Switzerland) operating at frequencies of 300.1 MHz (^{1}H) and 75.5 MHz (^{13}C). Each sample was packed into a zirconia rotor, sealed using a kel-f drive cap, and spun at 12 kHz. A CP contact time of 1 ms and relaxation delay of 5 s were employed, with 90° pulses of 3.5 and 4.5 µs used for ^{1}H and ^{13}C, respectively. All spectra were referenced with respect to Tetramethylsilane (TMS, 87920).

Peak areas were obtained using the automatic integration tool of TopSpin 3.6.1 (Bruker©, Fällanden, Switzerland), and subsequently normalised to relative areas and grouped into different functional groups according to the expected chemical shift regions for soil organic matter [163; 164]. These were as follows; alkyl C (0 - 50 ppm); methoxyl C (50 - 60 ppm); carbohydrate C (60 - 90 ppm); di-O-alkyl C (90 - 110 ppm); aryl C (110 - 142 ppm); phenolic C (142 - 160 ppm); carbonyl C (160 - 200 ppm). The percentage of each functional group was then calculated for the total of each FA.

2.3.11 Glasshouse FA vegetative growth and nodulation assays

Due to the legume yield effect measured in 2.3.2, FAs were tested in *M. sativa* for vegetative yield and nodulation rate. Treatments of VFA and MFA from 2.3.1, controls VC and MC from 2.3.7, and extra controls of no application (NA) and dH_2O were used.

Seeds of *M. sativa* cv. Daisy, Luzelle and Gea were sterilised, vernalised and germinated on water agar as in section 2.1.7. At 9 days post germination, seedlings were transplanted to 60 cell trays (individual cells were 4.5 x 4 x 14 cm dimensions) with Church Farm soil (see 2.2.4) in the glasshouse. At 12 days post germination, cells were randomly assigned to one of 8 treatments. Treatments were then designated a number from 1-8 by a lab colleague, Marco Fioratti (Metabolic Biology, John Innes Centre), and these given to N.C. making treatments blinded when treating plants and subsequent sampling; note NA treatment was not blinded due to practical necessity. Each cell received 1 mL of their specified numbered treatment.

For cultivar Daisy only, four soil samples for each numbered treatment were taken at both seedling transplanting (0 days post treatment) and biomass harvesting (21 days post treatment). Around 10 – 15 g of soil from treatment cells was mixed into 100 mL glass vials and stored at -20 °C.

For all cultivars at 21 days post treatment, individual plants above ~ 4 cm were harvested for each treatment (n = 6 – 12), and vegetative tissue dried in at 55 – 65 °C for biomass. The whole experiment was repeated separately for three individual replicates.

For cultivars Daisy and Luzelle, roots were washed with dH_2O to be free of soil. The numbers of pink and white nodules were scored visually with numbered treatment blinded.

Once vegetative biomass (all cultivars) and nodule count measurements (not including Gea) had been taken the results were unblinded. All treatment data was calculated relative to NA in Excel® 2016. Significance between treatments for p-value < 0.05 was shown with different letters using one-way ANOVA with Tukey testing in GenStat® 18th Edition. Graphs were made in RStudio.

As treatments were unblinded following the above measurements, so the origin of soil samples was known during subsequent assays.

2.3.12 Plate FA vegetative growth and nodulation assays

FAs were tested on agar plates either in sterile conditions or with *Sinorhizobium* inoculation. Seeds of *M. sativa* cv. Daisy, Luzelle and Gea were sterilised (0.5 g each), vernalised, and germinated on water agar as in section 2.1.7. Six treatment plates were prepared with 20 mL addition of treatments in 2.3.18 to a final volume of 200 mL FP media from 2.1.7 with 5 % agar No. 1 (Lab M, MC002). Two days post-germination 5 seedlings of the same size were transferred to each treatment plates. Half of the treatment plates were inoculated with 1 mL of *Sinorhizobium meliloti* 1021 culture prepared as in 2.1.7. Plates were sealed with micropore tape and transferred to CER for 21 days at 23 °C and a photoperiod of 16 h light (90 µmol m^{-2} s^{-1}) and 18 h dark.

At 21 days growth, total nodule number was recorded for both inoculated and sterile treatment plates. Seedling shoots and roots were then separately pooled for each treatment and dried 55 – 60 °C overnight. Biomass was measured the following day. The whole experiment was repeated in triplicate, and mean values calculated. Statistical analysis was performed as in 2.3.11.

2.3.13 *Sinorhizobium* colony forming unit counts

The effect of FAs on *Sinorhizobium meliloti* 1021 growth in cultures was analysed using single colony forming unit (CFU) counts. The strain was preincubated in 100 mL TY media for 2 days at 28 °C shaking at 200 rpm. The preincubated stock was diluted to 0.1 OD at 600 nm to add to treatment flasks.

Treatment flasks of 100 mL TY were set up as follows: NA = no addition; dH_2O = added 10 mL dH_2O; VFA/MFA = added autoclaved 10 mL of either 10 % VFA or 5 % MFA from 2.3.1 stock solutions; VC/MC = added autoclaved 10 mL of either 10 % VC or 5 % MC from Tables 2.10 and 2.11 solutions. Flasks were inoculated with 10 µL of preincubated stock and incubated at 28 °C shaking at 200 rpm. At timepoints of 0, 1, 2, 3, and 4 days dilutions from treatment flasks of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-}

^{7}, 10^{-8}, 10^{-9}, and 10^{-10} in 1 mL dH_2O were taken in triplicate. From these dilutions 10 µL was spotted out on TY agar plates, and plates placed upside-down in 28 °C oven for one day until single colonies had formed in a dilution spot at a rate of ~ 20 – 200. Plates were scanned using a Perfection V550 Photo scanner (Epson, SKU: B11B210301) and CFU recorded for dilution. An example can be found in Figure C6, Appendix C. Rhizobial cell density was calculated for dilution factor and total volume of culture at each timepoint. The whole experiment was repeated in triplicate to compare treatments, with graphs produced in RStudio and ANOVA performed using Genstat as above in 2.3.11.

2.3.14 Soil Microbial Phospholipid Fatty Acids Extraction

Soil stored at -20 °C from glasshouse *M. sativa* cv. Daisy assays in 2.3.11 were defrosted and mixed thoroughly. Each treatment (NA, dH_2O, VFA and VC) had samples from day 0 and day 21, in triplicate. Extraction of phospholipid fatty acids (PLFA) was performed using a modified protocol from Quideau et al., (2016) [165]. Prior to assays, a 5 g of soil sample was freeze dried (VirTis, Benchtop SLC model 4KBTZL-150, 2022131) in EtOH cleaned glass vials (used throughout) to remove all water from the soil. A negative control was also carried out without soil.

An internal standard of 250 µL methyl decanoate solution 1 % (299030) was added at the beginning of the extraction process. Total lipids were extracted using 4 mL Blyth-Dyer extract; 200 mL H_2HPO_4 50 mM, 500 mL MeOH. Extractions were vortexed for 1 min, then placed on an orbital shaker for 2 hours in the dark; PTFE tape was used to seal vials before plastic lids. Extractions were centrifuged at 1650 rpm for 15 mins at 4 °C, and supernatant was added to a new vial. The Blyth-Dyer extraction was repeated with the soil sample, and again supernatant added to vial. 1 mL chloroform and 1 mL dH_2O was added to the extraction, and the upper phase was aspirated with N_2 gas. The lower phase, which contained total lipids, was centrifugally evaporated in low boiling point mode at 60 °C and no heat lamp (Genevac EZ-2 Elite). Vials were stored overnight at -20 °C.

Elution of lipids was performed using SPE columns 500 mg 6 mL silica (SUPELCLEAN LC-SI, 57134) Columns were conditioned with 5 mL MeOH, and then 5 mL chloroform. Evaporated extracts were brought to RT, dissolved in 2 mL chloroform, and loaded into a column. A vacuum pump was used for elution at ~ 2 Hg. Neutral lipids were eluted with 5 mL chloroform, and glycolipids eluted with 5 mL acetone. With new vials underneath columns, PLFAs were eluted with 5 mL 5:5:1 MeOH:chloroform:dH_2O buffer. The upper phase was again evaporated using the centrifugal evaporator, with a N_2 gas purge.

Transesterification of PLFAs to produce fatty acid methyl esters (FAME) for detected was performed in a 37 °C water bath for 15 mins with 250 µL transesterification reagent; 0.561 g KOH dissolved in 75 mL MeOH, and 25 mL toluene added. At RT 400 µL acetic acid 75 mM and 400 µL chloroform was added. Phases were allowed to separate, and the bottom 300 µL of the bottom phase was added to a new vial. The transesterification process was repeated, with another 300 µL added to the new vial. Samples were again centrifugally evaporated until dry and purged with N_2 gas. Samples were dissolved in 75 µL hexane, and stored at -20 °C until ran on GC-MS.

For GC-MS analysis samples were ran using an Agilent GC-MS Single Quad Mass Spectrometer (7890/5977). Run information was as follows:

- Column: Zebron™ Inferno™ ZB5-HT column (Phenomenex®; length 30 m, internal diameter 0.32 mm, film 0.25 µm; Phenomenex Inc., California, USA).
- Carrier gas: Hydrogen at constant flow of 1.2 mL/min
- Inlet temperature: 250 °C
- Injection volume: 1 µL
- Injection mode: split–splitless (30:1)
- Oven temperature: Initial temperature 190 °C
  Ramp 10 °C/min to 285 °C
  Hold: 310 °C for 5 min
  Equilibration time: 0.5 min
- Auxiliary temperature: 280 °C
- Acquisition mode: SCAN between 50 - 500 m/z

- MS temperature: Source 230 °C
- Quad temperature: 150 °C

Analysis for FAME profiles from PLFA extracts was performed as below using MassHunter Profinder and Mass Profiler Professional.

2.3.15 Analysis of FAME profiles

FAME spectra from 2.3.14 were extracted from all chromatograms in MassHunter Profinder version 2.0 (Agilent B.08.00). The inbuilt wizard for batch recursive feature extraction was used to detect compounds with parameters as follows to avoid erroneous peaks due to noise: no region exclusion; noise cut 100 – 500; RT tolerance 0 – 0.1 min with minimum dot prod = 0.4; score(MFE) filter = 70; expected retention time 0.5 – 1.5 mins; Agile 2 Integration used with set parameters without smoothing; no peak filter; chromatogram format of centroid. Compounds in this wizard are identified with the NIST 17 library (NIST 17 MS Database and MS Search Program v.2.3).

The compounds identified were exported as compound exchange format files and imported into MassHunter Profiler Professional 14.0 (Agilent G3835AA). Again, the inbuilt wizard was used to determine fold difference of peaks with parameters set as follows; no abundance parameter; allowed tolerance of 30 s; normalisation algorithm set to standard as all spectra were similar. A compound list with abundances for each extraction was exported to excel for further analysis.

Most compounds found in all spectra were FAME as expected. Those not were low abundances of 2,6-Bis(tert-butyl)phenol, 1-Octadecanol, and 1-Dodecanol, and likely to be TMS derivative contaminants of the FAME, and thus discounted from analysis. The internal standard was Decanoic acid (C10:0) All identified FAME were assigned into their biomarker class for corresponding microbial community as compiled by Quezada et al., (2007) [166]. However, as some FAME biomarkers overlap in their assignments the final microbial groupings were reduced to ‘Common’, ‘Gram-negative

bacteria', 'Algae/Higher Plants', 'Gram-positive bacteria', 'Fungi', and 'Actinomycetes/Desulfobacter'.

FAME abundance in nmol g^{-1} was calculated for each methyl ester using its spectra peak area compared to the internal standard decanoic acid (C10:0, retention time 9.199) as:

$$nmol\ g^{-1} = \frac{methyl\ ester\ molarity \times \left(\left(\frac{area\ of\ methyl\ ester}{area\ of\ C10{:}0}\right) \times C10{:}0\ nmol\ L\right)}{total\ sample\ weight\ (g)}$$

Abundance values were used to calculate percentage of each microbial grouping in samples. Mean percentages of groupings were calculated with standard deviation in Excel, and Principal Component Analysis (PCA) plots created with ClustVis [167] using the NIPALS algorithm [168] within the pcaMethods Bioconductor package [169].

2.3.16 Soil microbial DNA extraction

Soil stored at -20 °C from glasshouse *M. sativa* cv. Daisy assays in 2.3.11 were defrosted and mixed thoroughly. Each treatment (NA, dH_2O, VFA and VC) had samples from day 0 and day 21, in triplicate. Total DNA from soil was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., QIAGEN Ltd., 12888-100) by the manufacturer's instructions with the following changes. Solution C1 was incubated to 60 °C for 10 minutes prior to use. A negative control with no soil was ran alongside the samples. A total of 1.5 g of each soil was used for extractions with 3 x 500 mg in sample preparation, cell lysis, inhibitor removal, and DNA binding steps. The triplicate extractions were mixed into one at the spin column and elution step. Final volume for samples was 200 µL.

Positive DNA controls for *Sinorhizobium meliloti* were also extracted. Cultures were grown in 200 mL TY media at 28 °C for 2 days at 200 rpm, as in 2.1.7.

DNA quality was measured on a NanoDrop™ 2000 spectrophotometer for a A260/A280 ratio of ≥ 1.7, and concentrations were recorded.

2.3.17 Soil DNA qPCR

Soil DNA extractions were used in qPCR for inferring any changes in *S. meliloti* dependant on treatment. Published primers used can be found in Table 2.12 below, with efficiencies confirmed between 90 – 110 % as in 2.1.10. DNA extractions were diluted to ~ 2 ng/µL, and qPCR set up as in 2.1.5 with DNA further diluted to 1:50 like cDNA. The qPCR programme was as used previously, but with the annealing temperature set to 56 °C and for a total of 50 cycles.

The transcript expression fold changes were calculated as in 2.1.5, with *S. meliloti* gene of interest *nodC* $2^{-\Delta CT}$ values calculated for individual reference gene sets, 799/1391 and Eub338/518, and expressed relative to experimental replicate one 'NA' treatment. The geomean was determined and was used to infer any differences in *nodC* expression in soil extractions between 0 day to 21 days depending on treatment. Graphs were made in RStudio.

Table 2.12: Primers used in study for soil DNA extractions for testing *Sinorhizobium meliloti* activity.

Gene name	Primer name	Sequence (5' – 3')	Source
nodC	SmnodC F	GCCGCTATCTCAATCTACGC	Trabelsi et al., 2009 [170]
	SmnodC R	TTGAAGCTGGGGACGATAAC	
799/1391	799 F	AACMGGATTAGATACCCKG	Beckers et al., 2016 [171]; Chelius and Triplett 2001 [172]; Walker and Pace 2007 [173]
	1391 R	GACGGGCGGTGWGTRCA	
Eub338/518	338 F	ACTCCTACGGGAGGCAGCAG	Fierer et al., 2005 [174]; Lane 1991 [175]; Muyzer et al., 1993 [176]
	518 R	ATTACCGCGGCTGCTGG	

2.3.18 Field trials with VFA

To assess if yield increases in *M. sativa* from VFA found in both plate and glasshouse experiments was applicable to growers, field trials were carried out over both 2017 and 2018 growing seasons. In 2017 trials were performed at Dengie Crops Ltd. (Southminster, Essex) with the help of Andrew Spackman (Farmacy Plc., Dorrington, Lincoln) in cv. Daisy and Fado. In 2018 the trials were at both Blankney Estates Ltd. (Blankney, Lincolnshire) and A Poucher and Sons (Bardney Dairies) Ltd. (Market Rasan, Lincolnshire) with the cv. Daisy and Gea respectively.

Treatments to be tested were NA, dH_2O, 1 % VFA (see 2.3.1) and 1 % VC (see 2.3.7). Individual experiment design of each plot is in Figure C9, Appendix C. Trials were designed as randomised block treatment plots for 2017 trials, and as randomised full trial treatment plots for 2018 trials. Each trial contained 4 - 6 plots per treatment of areas 4 - 10 m^2 with buffer zones between plots. As in the glasshouse trials in 2.3.11 treatments were applied and at 21 days post treatment samples were taken for vegetative biomass measurements using a randomly placed 625 cm^2 sampling quadrat with all tissue taken for biomass. Samples were also taken for protein and chlorophyll for 2018 trial plots as described in 2.1.13, 2.1.16 and 2.1.17. Statistical testing was carried out as in 2.3.11 for measurements, with ANOVA calculated in Genstat for significance between treatments.

2.4 FULVIC ACID RNA-SEQUENCING OF MEDICAGO SATIVA

2.4.1 RNAseq of VFA and VC experimental design

Due to the role *Sinorhizobium meliloti* inoculation plays in the growth effects of VFA, RNAseq was used to investigate transcriptional changes in the shoots and root separately. Figure 2.3 below shows the experimental design for RNAseq investigation with biomass measurements detailed below, with three independent replicates.

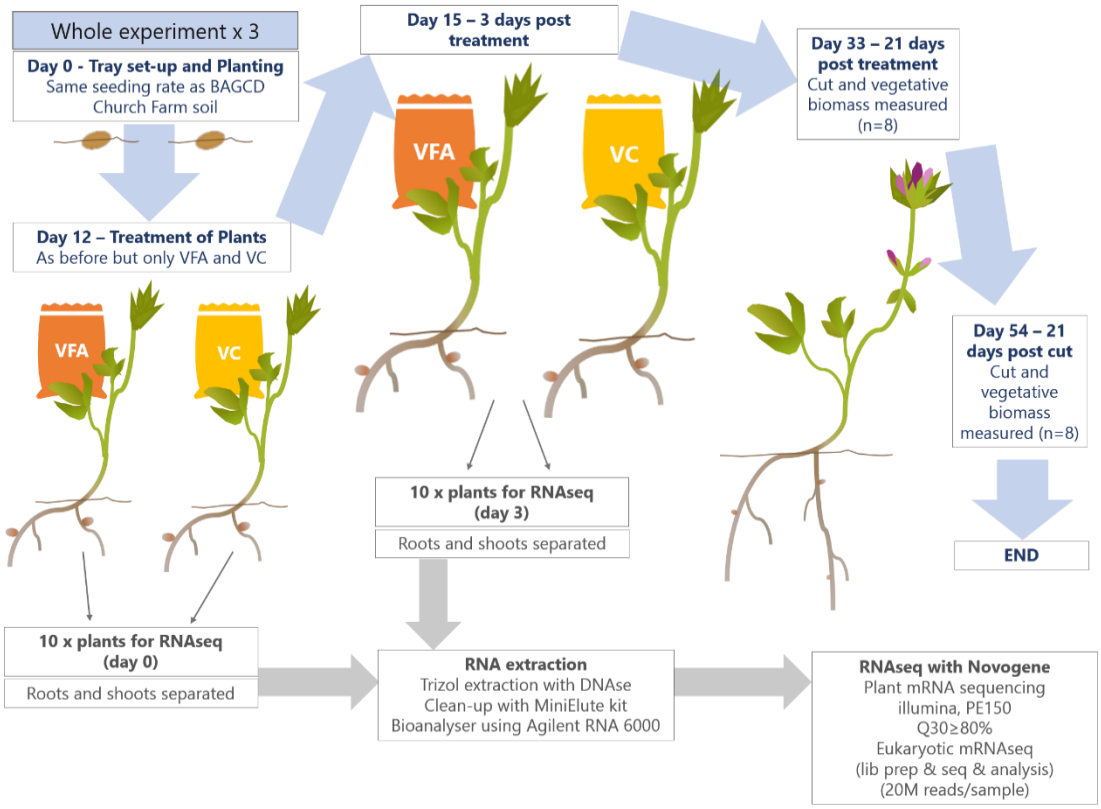


Figure 2.3: RNAseq experimental design for comparing transcriptional differences in shoots and roots of *Medicago sativa* cv. Daisy.

Treatments were either 1 % VFA or 1 % VC, with RNA sampling of tissue at day 0 and day 3. Vegetative biomass was measured as in 2.3.11.

2.4.2 RNAseq plant material and RNA extraction

Seeds of *M. sativa* cv. Daisy were sterilised as in 2.1.7. Seeds were sown in full seed trays (36 x 22 x 6 cm) containing Church farm soil as in 2.2.4 at a rate of 20 kg ha^{-1}, or 0.158 g per tray. Trays were watered every 3 - 4 days, and at day 12 were treated with autoclaved 1 % VFA or 1 % VC, described in 2.3.1 and 2.2.9 respectively. Plants were sampled for RNA at day 12 and day 15, each referred to as day 0 and day 3 in subsequent analysis regarding their treatment timepoints. For each sample ten plants were pooled, with shoot and root tissue separated. Therefore, each sample consisted of ten biological samples, and the experiment was carried out in triplicate over 3 days to provide three experimental replicates. Tissue was immediately frozen in liquid N_2 and stored at -80 °C until RNA extraction.

RNA extraction was carried out using the TRI Reagent (93289) method described in 2.1.2 with a final volume of 50 µL, and stored at -80 °C.

2.4.3 RNA clean-up and quality control

RNA from 2.4.2 was thawed on ice. Samples were purified using the RNeasy MinElute Cleanup Kit (QIAGEN Ltd., 74204) as per the manufacturer's instructions to a final volume of 30 µL; 25 µL was stored at -80 °C to be sequenced, and 5 µL was used to perform quality check using a Bioanalyzer and for any future qRT-PCR confirmation.

Quality check was performed with cleaned RNA samples using the Agilent RNA 6000 Nano Kit (Agilent Technologies, 5067-1511) as per the manufacturer's instructions for a total of 2 chips. This was ran using a 2100 Bioanalyzer Instrument (Agilent Technologies, G2939BA). RNA Integrity Number (RIN) and RNA concentration (ng/µL) were recorded. Samples with a RIN below 7 were checked with NanoDrop™ 2000 spectrophotometer for 260/280 to ensure RNA had not degraded during extraction. Any samples with a concentration above 500 ng/µL were diluted to be between 50 – 500 ng/µL with final volume of 23 µL sent for sequencing. General RNA quality check information and Bioanalyzer gel results can be found in Table D1 and Figure D1, Appendix D.

2.4.4 RNA Sequencing

Library construction and sequencing was performed by Novogene (HK) Company Ltd. (Hong Kong). Library construction was performed using Next® Ultra™ RNA Library Prep Kit (New England BioLabs Inc., E7530L) and sequenced on 1 lane of a HiSeq™ 2000 (Illumina, HWI-ST1276) in High Output mode using 150 bp paired end reads and V2 chemistry. Initial quality assessment of sample reads was performed using Phred quality scores with Illumina Q score calculations. Sequencing quality check information can be found in Table D2, Appendix D.

2.4.5 Read alignments

Read alignments were performed by Dr Jitender Cheema (Computational and Systems Biology, John Innes Centre) with aligned data to N.C. for all subsequent analysis.

Reads were aligned to two reference sequences for the close relative *Medicago truncatula*, the fully annotated A17 v.4.0 [177] and the first full assembly of R108 v1.0 [178]; A17 is a more annotated reference but *M. sativa* is more closely related to R108. Assembly used the R package [148] using Bioconductor [49] ballgown [179]. The mapping rate was 60-70 % for A17 alignment and 75 - 85 % for R108 alignment, with aligned transcripts totalling 57585 and 61019 respectively. Although read alignment was adequate in for both references it was decided that de novo transcriptome assembly would be more suitable for this case.

De novo transcriptome assembly was performed with Trinity [180], which used all samples, regardless of tissue, treatment, or timepoint to build. A total of 630599 transcripts were preliminary identified (including isoforms). BUSCO [181] was ran to check benchmarking of assembly using Universal Single-Copy Orthologs. BUSCO checked 430 conserved plant origin benchmarks and found that only 4 were missing from de novo assembly, providing a 93 % quality assessment score. Kallisto [182] was used to align assembly which is less subjective than ballgown mapping; Kallisto is a form of pseudoalignment, where compatibility of reads to

target are tested for likelihood, without a need for alignment. Pseudoalignment percentage scores can be found in Table D3, Appendix D. This provided both Transcripts Per Million (TPM) and Reads Per Kilobase Million (RPKM) for subsequent analysis.

2.4.6 Differential gene expression analysis

Differential gene expression was performed for both shoot and root tissue independently using Degust [183] and performed with all read alignments generated in 2.4.5. Sample multi-dimensional scaling (MDS) plots for 200 genes were generated to compare how experimental replicates clustered for different treatment and timepoints.

Tissue samples were grouped into treatment and timepoint for transcript analysis. Transcripts with both an absolute log fold change of 0.585 (1.5 x fold change) and a false-discovery rate (FDR) adjusted p-value (q-value) < 0.05 were considered as differentially expressed. The grouping of tissue samples requires all 3 experimental replicates to fit these criteria to be accepted, thus ensuring a very high benchmark was set at the beginning of analysis. Differential expression was checked using voom/Limma method [184] for log Fold Change (logFC) between treatments (VC, VFA) at both timepoints (0, 3); 0VC vs 0VFA and 3VC vs 3VFA. Differential expression was then check for individual treatments between timepoints; 0VC vs 3VC and 0VFA vs 3VFA. An example of this analysis for 0VFA vs 3VFA can be found in Figure D2, Appendix D.

To eliminate any differences caused by random chance or plant development changes over the 3-day timescale, transcripts that were truly differentially expressed based on the VFA treatment were calculated by subtracting 0VC vs 0VFA and 0VC vs 3VC from 0VFA vs 3VFA. It was this differentially expressed (DE) transcript list which was carried forward for the functional annotation.

2.4.7 Functional annotation

DE transcripts from degust analysis in 2.4.6 were imported into the Blast2GO v1.4.4 programme pipeline [185; 186] as FASTA contigs for functional annotation. DE transcripts were checked against NCBI's non-redundant NR database [187] with a BLAST expectation value cut-off of $1.0E^{-3}$, and hits excepted for no more than 20 sequences. In programme mapping was ran with the EMBL-EBI InterPro library [188] using amino acid mapping [189] with all families, domains, sites, and repeats available tested. Annotation of mapped results was ran using Gene Ontology Annotation Version 2019 [190; 191] with the follow strict parameters; Annotation cut off of 55; GO weight of 5 only; E-value-Hit-Factor restricted to $1.0E^{-6}$; Hit filter set to 500; Evidence Codes weighted from 0.5 to 1 depending on depth of evidence (default software parameters). To quality check once annotation was completed in Blast2GO a manual BLAST algorithm was performed [137; 138; 192] with NCBI database [193; 139; 194; 140] of the most significantly up or downregulated genes. Moreover, any genes lacking any GO annotation through InterPro library were checked for annotation in both QuickGO [195] and UniProt [196] and added to future analysis.

The inbuilt statistical wizard in Blast2GO was used to generate distribution graphs for sequences and hit species. Top GO annotation distributions and gene ontology directed acyclic graphs (DAGs) were also generated for molecular function, biological process and cellular components. Moreover, data was exported to Excel for manual bar charts containing all GO terms.

2.4.8 GO term enrichment testing

To test for enrichment of different categories of de novo *M. sativa* DE transcripts relative to all expressed transcripts found in *M. truncatula* (as the closest relative), the PANTHER Classification System v14.1 was used [197; 198]. GO-Slim graphs were generated for molecular function, biological process, and protein class, and then an Overrepresentation test was performed using the Fisher's exact test [199]. These were run

separately for up and downregulated DE transcripts in both shoot and root. Fold enrichment values and their p-value for each term was recorded.

2.4.9 Validation of RNAseq by qRT-PCR

Following RNA-seq analysis, qRT-PCR was used to measure expression of a subset of DE transcripts. From the root Overrepresentation test performed in 2.4.8, a subset of seven genes were chosen to confirm with qRT-PCR. Primers were designed for genes using available *M. truncatula* sequences, shown in Table 2.13, and primer efficiencies calculated as in 2.1.10. Primers with 90 – 115 % efficiencies were used in qRT-PCR.

Root RNA from 2.4.2 underwent cDNA synthesis as in 2.1.3. qRT-PCR was performed as in 2.1.5 using the reference gene *ACTIN2* in Table 2.6. Mean relative expression was calculated for experimental replicates and compared to RPKM logFC of DE transcripts.

Table 2.13: Primers used in study for *Medicago sativa* qRT-PCR confirmation of RNAseq analysis.

Gene name	Primer name	Sequence (5′ – 3′)	Source
Myb/SANT-like DNA-binding domain protein	Myb/SANT-like F	GGGCCACATCTGCAGTAGTA	Nicola Capstaff
	Myb/SANT-like R	TGCTTGGTGGGATGCTAAGA	
putative CC-NBS-LRR resistance protein	CC-NBS-LRR F	GCAACGCAACTTGAGAGAAT	Nicola Capstaff
	CC-NBS-LRR R	TTCCTCCAAACCCACAGTGT	
LysM domain receptor-like kinase 3	LysM kinase F	CGTCTACCTCCAATGAACAAGT	Nicola Capstaff
	LysM kinase R	GTGTCTTCCCTGCAATGACTAC	
MtN6	MtN6 F	GAAGAGTTCGGGGAATGCAT	Nicola Capstaff
	MtN6 R	CGTCCTCTCCTATTAATTGGCG	
nodulin-26	nodulin-26 F	TCAACCAGAACCAGAGCCAT	Nicola Capstaff
	nodulin-26 R	ACCTCATCACAGCCCTAGTT	
NRT1/ PTR FAMILY 4.5-like	NRT1/PTR 4.5 F	TTCTTTGCTCGTCCTAGGGG	Nicola Capstaff
	NRT1/PTR 4.5 R	CGACGACAATGGAGAGCAAG	
beta-tubulin subunit	beta-tubulin F	GAATTGTGATTGCTTGCAAGGT	Nicola Capstaff
	beta-tubulin R	TCATTAACCTCTGAATCGTCACG	

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Chapter 3: Marker genes in *Festulolium* can be used to assess crop and soil N status, to aid with grower decisions to improve future yield

3.1 INTRODUCTION

3.1.1 Soil N status affects plant NUE and yield

Forage NUE is an important target for increasing forage production, particularly as the protein content of crops is so valuable for animal feed. Protein accumulation is linked to nitrogen (N) status and when the N supply is supra-optimal greater storage occurs. Forage crops often require N in greater amounts due to their increased growth, storage capacity and higher fibre content [200], when compared with grain crops that have been bred for high seed yield and starch content. For forage crops, NUE is the accumulation of vegetative tissue (biomass) harvested compared to the N fertiliser inputs. Principally NUE for forage crops can be based on N utilization efficiency (NUtE) and N uptake efficiency (NUpE) as we are interested in the highest achievable biomass of the shoot and leaves which will form the content to be dried for feed production [72]. Not only does this include biomass, but also the relative N levels in this tissue; it is not enough to only have a high shoot biomass, the crop must also yield optimal amounts of protein N. This total N within the plant is in turn important for the quality of the final animal feed. Moreover, when looking at the effect of fertiliser use, we are interested in how both the biomass and N status change on application and thus NUpE.

Nitrogen availability to animals is predominantly from forage proteins and these are estimated using crude total protein Kjeldahl measurements. Protein is abundant mainly as RuBisCO, with tissue specific proteins such as prolamins within monocot seeds [201; 202], although relative amounts do vary between forage species [23]. This is especially true when comparing the relative contents in grasses such as *Lolium* spp., *Festuca* spp., and *Festulolium* hybrids, with herbaceous legumes particularly lucerne (*Medicago sativa*), which is grown widely in the UK due to its high protein value [22].

Forage crops offer challenges for NUE as there is a requirement for optimal yield of shoot biomass with a high N content (NUtE) while also optimizing N fertilizer acquisition (NUpE) throughout the growing season [203].

Studies have already shown, through ^{15}N labelling of *Lolium perenne*, how deficiency caused by low N fertiliser application causes an increase in the protein substrate pool whereas the store pool decreased in size and turnover rate [204]. It is important to maintain the N status of the crop throughout the growing season, depending on external abiotic factors, to ensure optimal production. In short, soil N supply must be optimised for individual crop growth [205]. This explicitly highlights the importance of fertiliser studies which assess soil and plant N status, for consequent N composition of forage crop vegetative tissue. Additionally, the intense cutting regimes, or grazing, within the management practice of cultivating forage crops exacerbates this challenge [49; 50; 206].

As NUE in plants is strongly associated with soil N status, many plant genes relating to N acquisition or metabolism may be useful to investigate this link. Genes which are important in carbon metabolism may be of relevance also, due the relationship of C:N for optimal plant growth [207-209]. Moreover genes related to the storage of proteins have been identified in both aboveground [210; 211], and belowground [212-214], tissue, which may be good candidates for exploring. Such genes will be discussed below but many references will be the homologues in *Arabidopsis thaliana* due to their better characterisation.

3.1.2 There is a wide variety of genes associated with NUE

As mentioned above, many genes associated with NUE have been well characterised in the model plant *A. thaliana* [117]. These *A. thaliana* genes, alongside more limited progress in grass research will be discussed below. Resources such as the published draft genome sequences of *L. perenne* [215] and *Brachypodium distachyon* [216] will make future analysis possible in grasses and other forage crops. The *NRTs*, *GS* and *NADH-GOGAT*, *RBCS* and *VSPs*, and *TIPs* gene families will be discussed with their characterisation in model species including *A. thaliana*, as well as any grass or legume related advances.

Nitrate transporters (*NRTs*)

Plants have evolved two nitrate uptake systems using proton-coupled transporters due to the high variability of available NO_3^- which is affected by soil type, day length and season. Because of their importance, these two nitrate transport systems must be considered when investigating genes associated with NUE. The high affinity transport system (HATS) has transporters with a K_m ~ 50 μM for NO_3^-, whereas the low affinity transport system (LATS) transporters have a K_m of ~ 5 mM. Examples of HATS and LATS transporters in *A. thaliana* are the *NRT2* and *NRT1* gene families [217]. Transporter kinetics were often identified using *Xenopus* oocytes assays or using mutant plants lacking expression of a gene [218]. An NRT of particular importance is *NRT1.1*, referred to as *CHL1*. *CHL1* encodes a protein that can operate as a dual-affinity transporter in both HATS and LATS ranges [219]. *NRT1.1* has become accepted as the primary nitrate sensing transceptor affecting the expression of numerous genes including *NRT2.1* under different conditions. This regulation extends to post-translational phosphorylation at T101 by a CIPK23 [220; 221], with the phosphorylation status being the basis for its affinity changes due to structural flexibility [222]. The *Medicago truncatula NRT1.3* has become accepted as the legume equivalent of a dual-affinity transporter [223]. Other examples of *NRTs* have been found across monocot cereal species [224-226] and are related to forage grasses.

There is redundancy in the N uptake system, as with many nutrient uptake pathways. For example, in *A. thaliana* when *NRT2.1* is knocked-out, plants show partial compensation through increased expression of *NRT2.2* with restoration of shoot:root N ratios to wildtype levels [227]. The ability of these primary transporters to affect root architecture is a key to their importance. *NRT2.1* initiates lateral root growth, *NRT1.1* is required for root primordia formation and emergence [228]. Alongside transcription factors such as the MADS-box member *ANR1*, roots are able to stimulate or initiate lateral root growth by sensing not only the levels of N but also in which form the N exists [229]. Control of root architecture is also affected by phytohormones, predominantly cytokinins, abscisic acid and auxin. Cytokinins and abscisic acid repress N uptake and NRT activity, and

auxin stimulates lateral root formation and therefore indirectly uptake [230-232]. Moreover, the interaction with NRT2s and N uptake requires extra genes such as *NAR2* to carry out function across many species [233]. Members of the *NRT1* family in *A. thaliana* have been shown to be involved in the translocation of NO_3^- from roots to shoots and vice versa [234-236]. The source-sink dynamic of N distribution is mediated by a complex regulatory exchange between the xylem and the phloem. This intricate network with complicated regulation, makes the study of *NRT* families a important line of research for plant scientists. Despite the advances still being made in this area in *A. thaliana*, and the accepted hypothesis that many other plant species will use a similar system, there remains a lack of characterised examples to study in forage crops. Plett et al., (2010), highlighted how the use of phylogenetic analyses in grasses to investigate *NRTs* is challenging, especially with the *NRT2* family [237]. Since then a flurry of research has shown advances in the field [238-244], however the *NRT* network in grasses largely remains elusive.

While the *NRT* family may be an interesting case for candidate N status genes in the future, for now it may be prudent to analyse more downstream genes related to assimilation and storage of N as proteins, rather than genes required for N uptake and distribution.

Nitrogen assimilation (*GS* and *NADH-GOGAT*)

Once the root architecture has developed to adequately uptake NO_3^- which heavily depends upon the *NRT* network, the N needs to be uploaded into vacuoles or transported to plastids for movement from the root to the resulting vegetative tissue [245]. As N assimilation is energetically costly it is important that this process occurs close to the photosynthetic apparatus where energy is more highly and quickly available [246]. Nitrogen assimilation from nitrate requires reducing power, usually from photosynthetically derived NADPH, in both leaf and root tissue, thus highlighting the complexity of the interactions between the C and N cycles. Carbon fixation provides not only the reducing power for N assimilation, but also the substrates of the GS-GOGAT cycle for amino acid synthesis through ammonium assimilation. In accordance with this, N availability has

been shown to greatly affect metabolic profiles in *L. perenne* [247] and *L. multiflorum* [248]. Genes related to the GS-GOGAT cycle and NADH production have been recently examined in a range of forage crops; *Glutamine synthetase* (*GS*) in grasses [249] including *Lolium* [146; 250; 251], and legumes including *Lotus japonicus* [252], *Glycine max* and *M. sativa* [253-255], and *M. truncatula* [256; 257]; *NADH-dependent glutamate synthase* (*NADH-GOGAT*) in the grasses *Lolium* [258-260], *Eleusine coracana* [261] , *Phragmites australis* [262], and legumes [263] including *M. sativa* [264] and *M. truncatula* [265]

Nitrogen reduction (*NR* and *NIR*)

After N has been acquired by the plant it then requires assimilation into amino acids. The assimilation pathway includes the key enzymes of nitrate reductase (NR) and nitrite reductase (NIR) [266]. These NR and NIR enzymes are able to reduce N sources for amino acid biosynthesis (predominantly the major amino acids are glutamine, glycine, serine). The complex interaction of N metabolism with carbon metabolism means that the reducing capabilities of the cells are affected by diurnal conditions [267]. Illumination of leaves increases the transcript levels and post-translational activity of both NR and NIR [268], leading to an accumulation of reduced N, which in turn will be converted to the aforementioned amino acids through the day via the GS-GOGAT cycle [269]. This flux in metabolism leads to depletion of stored N and so acquisition of N through the roots is affected. Furthermore enzymes found in the Krebs cycle (e.g. phosphoenolpyruvate carboxylase, citrate synthase, NADP-isocitrate dehydrogenase) are also upregulated to keep up with the increased metabolic output [269]. As the Krebs cycle receives its carbon source from pyruvate produced by glycolysis from the breakdown of hexose generated from photosynthesis, it is easy to see how such an increase of NR and NIR activity can cause an upstream effect on the whole metabolic function of the plant. Because of this, both NR and NIR proteins are regulated by light signals, and the N cycle transcription factor *HY5* [270], with NR being regulated further by reversible phosphorylation [268; 271; 272].

The activity of the NR and NIR enzymes, in conjunction with the accumulation of amino acids (predominantly glutamine), can give a representation of overall plant metabolic rate [273]. Moreover, studies in *Lolium* [274-277] and *Medicago* [253; 278-280] has been well-covered in the literature like with *GS* and *NADH-GOGAT* previously, and making the *NR* and *NIR* genes good potential candidates for future study.

Protein storage (*RBCS* and *VSPs*)

Upon assimilation N can be stored in the cell. As grains and fruits are of more widespread agricultural significance there has been a comparatively greater research focus in seed storage of proteins and grain filling. However in terms of forage crops it is important to consider the vegetative storage of protein, as the crop is cut usually prior to flowering. There are two main N storage complexes to note here, RuBisCO and vegetative storage proteins (VSPs), requiring the genes *RBC* (large *RBCL* and small *RBCS* subunits) and *VSPs* for their formation.

Around 50 % of the total leaf soluble proteins are found in the chloroplast in the form of RuBisCO [281] encoded by the *RBC* genes [282; 283]. RuBisCO is the key enzyme in carbon fixation and is an active N protein continuously being regenerated in green tissues. It also shares the same pattern as VSPs where higher amounts are accumulated in N surplus and lower amounts in times of starvation [284]. As RuBisCo is found in the chloroplast it does not require actual storage compartments. Due to their role in photosynthesis, *RBC* genes have been studied across the grass family [285-288].

In 1983, Wittenbach first characterised a VSP whose accumulation increased in leaves even after pod removal in soybean [289]. Since then, the prevalence of VSPs as a main mode of protein storage in many crops has been realised [289; 290]. Soybean has two VSPs of sizes 27 kD and 29 kD; *Arabidopsis* has been shown to also have two homologues of these proteins [291]. Work in both these species has shown *VSP* expression to be regulated by jasmonic acid pre-empting its role in stress responses, a likely characteristic of a storage entity. The breakdown of VSPs can be used to cope with the shortening days and colder environment of winter or

be used after herbivory attack to increase substrates to repair the tissue affected.

Found in leaves, stems and seed pods, VSPs that are strictly glycoproteins show increased accumulation when N is in abundance; these VSPs can also be rapidly degraded on N-depletion [292; 293]. Work has shown that their accumulation and depletion could make them a marker for the cessation of N uptake, such as in the senescence of older leaves [294]. *VSP* genes have been particularly well studied for many decades in *M. sativa* [295-299] with limited work in grasses [300-302].

VSPs and nitrate are stored within vacuoles. It is generally accepted in current plant physiology that plants possess two distinct types of vacuoles, although care should be taken since this may not be universal [303]. These two types are termed lytic vacuoles (LVs) and protein storage vacuoles (PSVs), and can occur individually or in combination in a cell [304]. Lytic vacuoles have been likened to the animal lysosome, an organelle containing hydrolytic enzymes to break down molecules for remobilisation and the storage of cell components. Conversely, PSVs are viewed as the storage capacity of cells with roles including turgor pressure of tissue, ion sequestration, secondary metabolism and, as the name suggests reserve protein accumulation. In terms of forage crops it is the PSV type that is of significance for research to improve NUE. Nitrate is stored within such vacuoles with the help of CLC transporters [305], which could be another candidate marker gene for NUE studies.

The trafficking pathway is highly conserved across all eukaryotes [306] and should be viewed as a highly regulated system rather than a passive flow which ends in vacuole formation. The Golgi apparatus is the main site of regulation and its sorting capacity later determines the type of vacuole produced [307]. This organisation is caused by the vacuolar sorting determinant (VSD) present [308]. These sequence motifs act as signatures and direct the Golgi-derived protein to vacuole formation rather than secretion to the cytoplasm, with a lack of receptor leading to mis-sorting [309]. The major difference in sorting to either vacuole is the use of either clathrin-coated vesicles (CCVs) as in LV formation or dense vesicles (DVs) as for PSVs [310; 311].

Although these vacuoles have differing trafficking pathways and characteristics, it is thought that their status in cells can change due to external effects. For example removing the shoot tip of young soybean plants can push LVs into PSVs, as measured by *tonoplast intrinsic protein* (*TIP*) gene expression [312]. The opposite change has been shown to occur in meristematic cells of germinating tobacco seedlings [313]. In other cases vacuoles with both LV and PSV characteristics have been found in young barley and pea seedlings [314]. For older tissue as with mature vegetation in forage crops the nature of the vacuole is likely to be more defined [315]. TIPs are discussed below.

Vacuole protein pathway (*TIPs*)

Aquaporins (AQPs) are a diverse family of transmembrane pore proteins [316] whose roles are well documented as regulating the transport of a range of substrates including water, gases such as carbon dioxide and ammonia, metalloids and more complex molecules such as organic acids. Maurel *et al*., (2015), concludes that AQPs are so widespread in their roles of regulating a vast array of key processes such as lateral root development and turgor pressure control of tissues that they are becoming integrated into multiple fields [317]. Different AQPs are localised to plants at specific cellular membranes, and at various times of development. Tonoplast intrinsic proteins (TIPs), are pore proteins with a distinct role in vacuoles (bounded by the tonoplast), and therefore an essential part of the secretory pathway. The localisation of a TIP to the tonoplast and its function as a specific water transporter pore was shown in 1993; TIP1.1 mRNA expression in *Xenopus* oocytes induced high membrane permeability [318]. Since then, TIPs have long been investigated for their designated association with different vacuoles with some shown to be linked to PSV [319-321]. Research with resurrection grasses [322-325], have led to more insight into how *TIP*s may function in forage species [146].

Identification of different families of NUE genes provides a platform to choose potential forage N status marker genes. These could be used to

determine N content of tissue more rapidly than conventional testing, with potential insights into soil N status.

3.1.3 Plants marker genes for a variety of parameters

Forage crop breeding programs are fraught with difficulties, due to individual plants having high genotypic and phenotypic heterogeneity in a cultivar. Moreover many species are polyploid, a problem that is exacerbated within hybrid grass populations. Few agronomic traits are linked to a distinct gene or number of genes [326; 327]. Recent studies have focussed on specific legumes [328-332] and grasses [333-335; 332; 336; 337], which can provide a wealth of potential genes of interest for NUE similar to, and including, those described in the last section.

One of the most prominent breeding successes in forage crops was the exploitation of closely related species of *Lolium* and *Festuca* to create hybrid *Festulolium* cultivars [338; 339; 13; 3; 340; 341]; backcrossing using conventional breeding methods of *Festulolium* generated novel hybrids with more stable protein content when compared to parental lines. Advances in phenotyping may make it easier to quantify of characteristics in the breeding programmes, such as high level imaging of crops to determine later traits especially biomass [342].

Additionally, new forage cultivars are being generated through advances in transcriptomic data with more sequences available for breeders to exploit [343-347]. This has included the identification of SNPs in *Lolium* [335; 348] and *Trifolium* [349-351] which may be used in programmes to improve on breeding of new cultivars. Moreover, draft genomes for forage crops are increasingly common [352-355; 346; 356-358; 215; 359-361; 348; 337]. There are many advances using such resources [18] including some in *M. sativa* where QTL and gene analysis have been used to investigate lodging resistance and vigor [362], plant height and regrowth following harvests [363], flowering and stem height [364], and drought tolerance [365]. *Lolium* has similarly been investigated with improvements in biomass increases [366; 367] as well as tracking of such phenotypic traits in programmes [368]. In addition many potential genes have been

identified in model plants that may become the targets for gene editing techniques [369; 370; 336], or the focus of TILLING populations [371; 372; 332], in forage crops.

Despite the above advances within breeding systems, there is a lack of the direct use of genetic information by the grower. Although, new cultivar breeding is crucial for the industry to progress in terms of both yield and efficiency terms, there is a huge wealth of genetic information that might be used by the grower themselves.

A genetics approach assessing crop efficiencies during growth could be investigated for multiple forage species, with comparison to current testing methods. This method could test the expression pattern of NUE related genes to see if these can be used to infer plant nutrient status, particularly as N status marker genes. These may also give information about soil N levels. Soil N is likely to be the biggest factor affecting crop yield, with the next important factors including pathogen responses and drought tolerance. Having one measurement to accurately assess both crop and soil N status may offer the prospect of future yield estimation or a guide for changing management practices to improve yields. The current N status testing methods of both crop and soil are discussed next.

3.1.4 Current testing for soil N lacks spatial and temporal detail

There are very few measurements of NUE in forage crops [373; 374]. At present forage growers cannot easily and reliably determine the N status of their crops. Most commonly used methods are biomass recording at the end of production periods (at cutting times for forage crops), Soil-Plant Analyses Development (SPAD) measurements for chlorophyll, or costly plant tissue nutrient analysis including protein assessments using the Kjeldahl method. The first method of biomass recording is passive, and information can only be used for next cultivation periods, whilst the second two are time-consuming, costly, and may have variable results based on how samples are collected and assayed. Plant samples can only be taken periodically and must be done carefully to increase the accuracy of

measurements across a whole site. Some research has focused on the use of spectral data to evaluate crops [375], but such techniques require further investigation and can give false readings caused by pathogen attack. It may be better if the farmer could determine the crop N status directly and then make an informed decision as to how they should subsequently fertilize the plot. This would enable more efficient targeted fertiliser use, thus increasing forage biomass with lowered costs and is the underpinning aim of precision agriculture. Better strategies of crop testing should be developed to reliably inform the grower of nutrient status to improve NUE efficiency.

For maximum biomass production, it is important to maintain the N status of the crop throughout the growing season and this requires an optimised soil N supply [205], particularly in coordination with cutting regimes. Furthermore, application of too much N fertiliser results in wasteful run-off, and sub-optimal supply results in decreased biomass production; Inefficient fertiliser use is of environmental, agricultural production, and economic concern. Maintaining N supply for maximal yield is limited by two factors: 1) an easy, consistent measure of the crop's status and 2) reliable and reproducible tests for soil N levels [376].

Presently farmers, or fertiliser suppliers, take limited samples of soil across their growing area in the hope that this is representative of the N in the whole plot through the growing season. Not only is the representative N level tested for a field usually spatially limited with only a small section being measured, it is also temporally lacking as costly soil analysis is not performed on each field each year. For example, a grower with a large farm may only have the resources, time and funds to test the N level of each field every other year, at best. In addition, although soil N level is strongly linked to plant production, it may not always be the best indicator of a plant's N status a useful measure of NUE.

3.1.5 Aims of this chapter: Testing N status marker genes in forage crops

This chapter describes work investigating the development of N status marker genes in the forage crops *L. perenne*, *Festulolium* and *M. sativa*. This may be useful for forage growers to assess both their crop's growth, as well as the soil N status. These techniques may be used in breeding programmes. Such method development can be tested in the glasshouse, and then verified in the field to decide on the suitability of its use.

To assist in the choice of N status marker genes to test, there is scope for the use of the molecular markers outlined above, with the future possibility of a PCR test for the optimal time of harvest based on the expression of candidate genes like storage proteins. Such tissue testing of crops can also be used for decisions on the timing of fertilizer applications as the two evaluations can be made around the same time. There is scope to identify a suite of marker genes to help decide when these key decisions are made. The aim was to identify if such a suite could be used in prominent UK forage species in the glasshouse alongside conventional testing methods. This would then be trialled in the field to see if such a method is utilisable by forage farmers.

3.1.6 Materials and Methods

The methods used in his chapter are detailed in Chapter 2, in section '2.1 N STATUS MARKER GENES'. Supplemental data is provided in Appendix A.

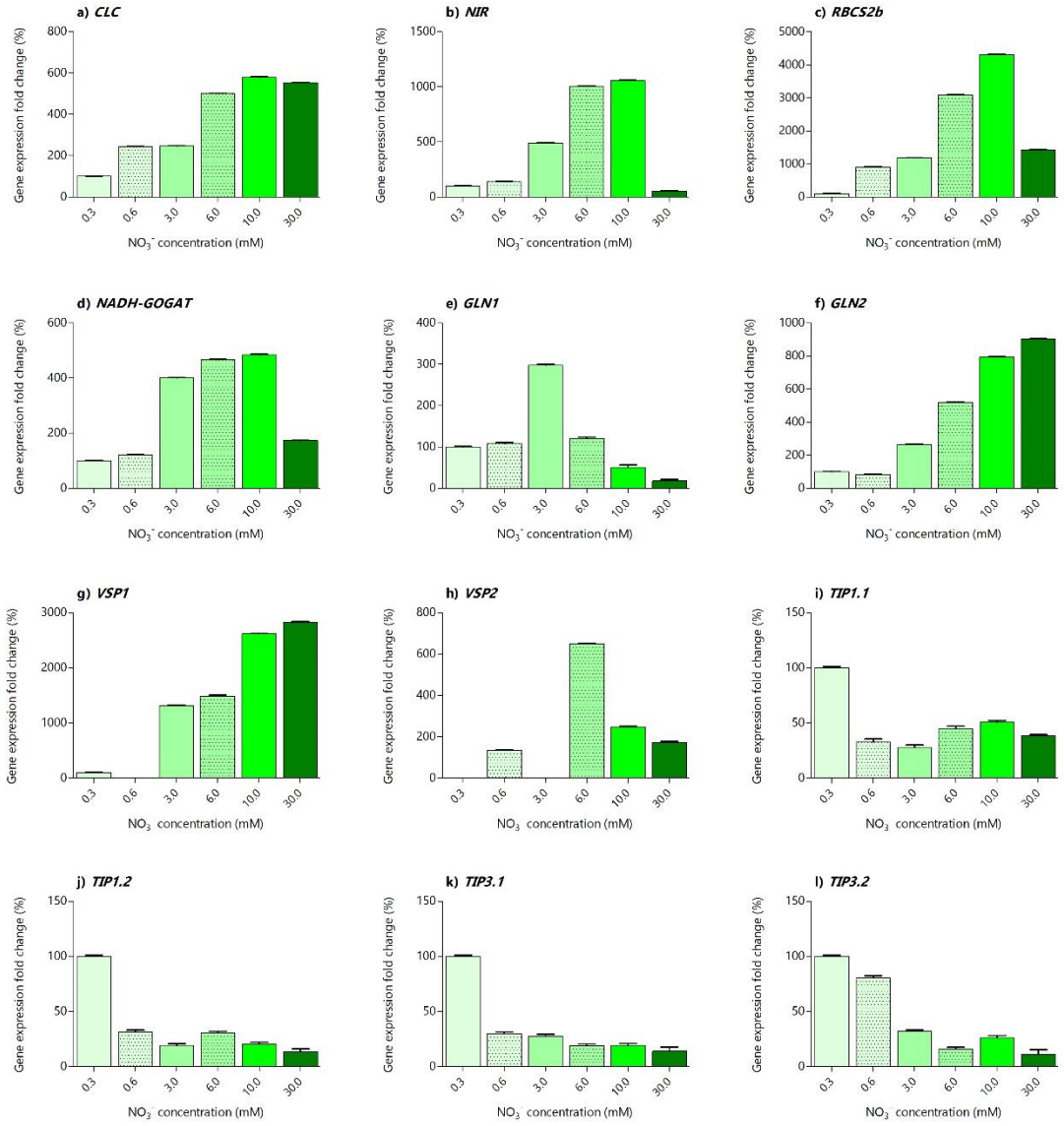
3.2 RESULTS

3.2.1 Nitrogen status candidate genes have different expression patterns in *A. thaliana* in relation to NO_3^- supply

A literature search was undertaken to find suitable candidate genes for N status in forage crops. The search began with considering *A. thaliana* genes with good characterisation and included those known to be important in C:N metabolism, or vacuole transport. The *A. thaliana* genes were also checked for expression with parity across vegetative developmental stages using the *Arabidopsis* eFP Browser [130] (Figure A1, Appendix A).

Expression pattern testing of these 12 candidate genes with *A. thaliana* was carried out under different NO_3^- treatments. Nitrate was used as the N source as usually it is the most abundant soil form of N for plants, whilst also being relatively easy to control compared to other N sources. Individual candidate genes were shown to have different patterns of gene expression under NO_3^- response (see Figure 3.1). Most of the genes showed an increase in relative expression with increasing NO_3^- concentration, including *CLC* (Figure 3.1 a), *GLN2* (Figure 3.1 f), and *VSP1* (Figure 3.1 g). Many others also increased in their expression, however reached a peak at 10 mM NO_3^- with a drop in relative expression fold change at 30 mM; see *NIR*, *RBCS2B*, and *NADH-GOGAT* (Figure 3.1 b, c, and d respectively). This suggests that for *A. thaliana* the optimal concentration of NO_3^- for growth is below 30 mM, and above this point there is a negative impact on growth.

Moreover the results showed that these effects are independent of the cation present in NO_3^- treatment, as expression patterns for KNO_3 tests were equivalent to $Ca(NO_3)_2$. All *TIP* genes showed a decrease in their expression fold change in relation to increasing NO_3^- concentration, see Figure 3.1 i)-l). This assays shows how a number of genes all affected by N status of environment can have different patterns of expression. This variation in expression pattern will be investigated in forage crops.



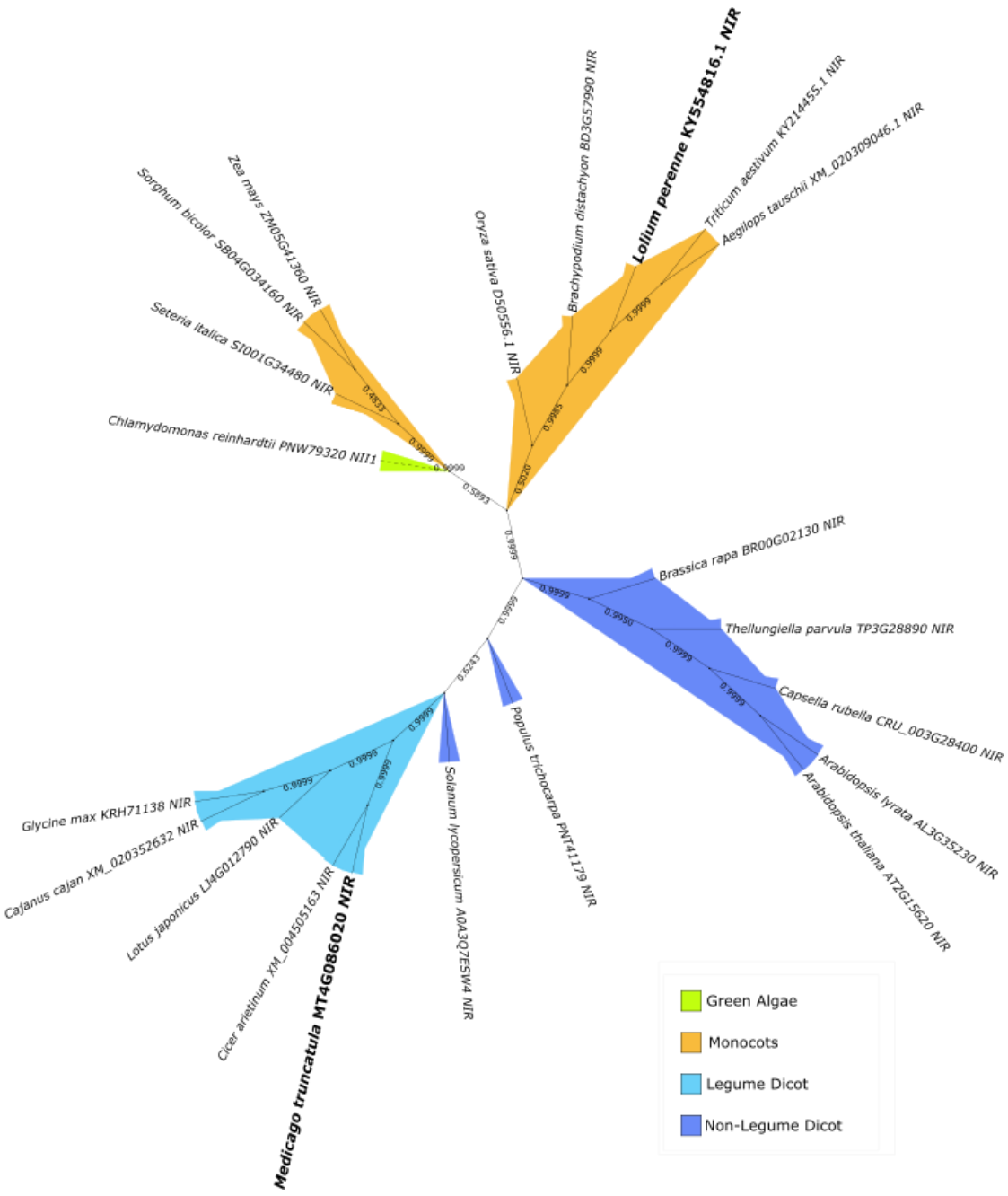
<u>Figure 3.1:</u> Gene expression patterns of candidate N status marker genes in *A. thaliana* vegetative tissue after three weeks of growth.

Expression was calculated as the geometric expression of independent $Ca(NO_3)_2$ and KNO_3 experiments relative to lowest NO_3^- concentration (0.3 mM shown as 100 % in pale green). Bars are denoted for increasing concentration using darkening shades of green, and error bars are standard deviation of expression using two reference genes with 12 biological replicates, in two independent experiments. Expression patterns of genes varied dramatically in relation to NO_3^- concentration; a)-h) shows those genes which are generally upregulated with increasing NO_3^- concentration; *CLCa*, *NIR*, *RBCS2B*, *VSP1*, *VSP2*, *GLN1*, *GLN2*, *NADH-GOGAT*; i)-l) shows genes downregulated; *TIP1.1*, *TIP1.2*, *TIP3.1*, *TIP3.2*.

3.2.2 Model species sequence alignments can be used to investigate forage crops

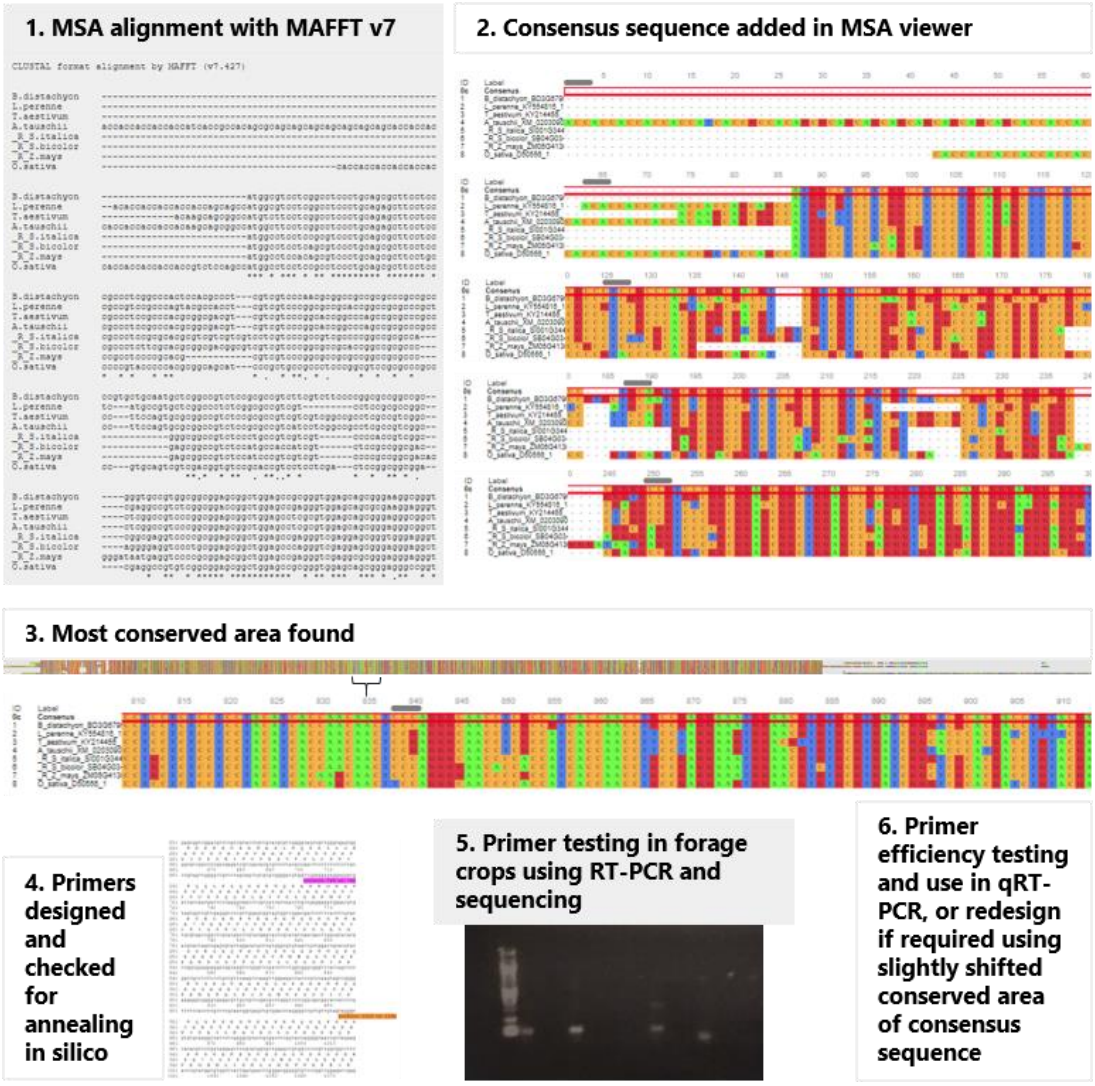
Through compiling known and predicted leguminous dicot and monocot gene coding sequences it was possible to determine which candidate N status marker genes tested in *A. thaliana* (Figure 3.1) could be closely related to forage crops with a similar sequence for use. A phylogenetic tree was built using the compiled sequences can be seen for *Nitrite Reductase (NIR)* sequence alignment in Figure 3.2 below. Comparing known and predicted sequences for related species is important as many forage crops including *M. sativa* and *L. perenne* (shown in bold in figure), and *Festulolium* hybrids do not have fully annotated genomes published at present. Candidate genes with a high degree of sequence similarity to genes fully or partly characterised in model species were more likely to be usable in these crops. The candidate genes with high sequence similarity by nucleotide alignment were *NIR*, *GLN*, *RBCS*, *NADH-GOGAT*, and *TIP1* (*MCP1* in *M. truncatula* homologue for legume sequences). Reference genes were also compared across species with alignments.

Consensus regions from sequence alignments was used to develop primers to test with forage crops in glasshouse conditions. Figure 3.3 shows a pipeline for using the high similarity sequence alignments to find conserved areas to design qRT-PCR primers. The pipeline was used for grasses and legumes separately to generate primers to test the forage crops found in Table 2.4 and 2.5. These primers were tested using PCR to check for predicted band sizes, as shown in Figure A2, Appendix A. The percentage of correct size bands for species indicated how well the conserved sequence area used for primer design was actually conserved across forage grasses and legumes; grass primers were ~ 56 % utilisable in species tested, and legume primers were ~ 71 %. Correct expected size bands were sequenced for both *M. sativa* cv. Daisy and *L. perenne cv. AberMagic* to check sequences matched the expected genes. Primers were checked for annealing efficiencies between 90 – 110 %. These N status marker primers were for the genes *NIR*, *RBCS*, *NADH-GOGAT*, and *TIP1* in grasses, and *NIR*, *GLN*, *RBCS*, *NADH-GOGAT*, and *MCP1* in legumes.



<u>Figure 3.2:</u> Phylogeny of temperate forage and related model species for *Nitrite Reductase* (*NIR*).

Species included were many model dicots including *Arabidopsis thaliana* shown in blue, with those that are legumes highlighted with light blue. Monocots were displayed in orange, and *Chlamydomonas reinhardtii* with *NII1* was used as an outlier. The model species *Medicago truncatula* and *Lolium perenne* with interest for forage crop research are shown in bold.



<u>Figure 3.3:</u> Pipeline for candidate gene primer design for forages crops.

Primers to test in forage crops were designed as follows; 1. Multiple sequence alignment was built using MAFFT v7 [142] for a gene using available and predicted legume or monocot sequences (in the above example this is *nitrite reductase* nucleotide coding sequences in monocots closely related to forage grasses); 2. A consensus sequence was added with MSAViewer [144]; 3. Most conserved section of sequences identified; 4. Primers were designed in silico [145; 127; 125; 126]; 5. Primers were tested with RT-PCR for a range of relevant species; 6. Primers for conserved consensus area were tested for efficiencies and used in future analysis of forage crop.

The N status marker gene primers were tested using qRT-PCR in forage crops grown under different NO_3^- treatments, like with *A. thaliana* in Figure 3.1. The forage crops tested were the grasses *L. perenne* and *Festulolium*, and the legume *M. sativa* (cv. Daisy and Luzelle). These species were chosen due to their high yield and common use by UK growers (including BAGCD members), as well as their prominence in temperate forage research globally. If a strong relationship between gene expression and plant N status can be found within glasshouse grown forage crops, then in-field sampled tissue could also be tested.

3.2.3 N status genes also showed different gene expression patterns in grasses in relation to NO_3^- supply

Gene expression was tested at three weeks after the NO_3^- treatment. At six weeks the total vegetative yield was measured as the aboveground biomass. Grasses were tested under a range of nitrate concentrations like *A. thaliana*, to mimic potential differences in supply occurring during the growing season that result from management practices. For example, if soil acidity is a problem a grower may add less N fertiliser in some fields, causing a likely drop in soil NO_3^- concentration. As *M. sativa* is a nodulating legume it was only tested under a small range of NO_3^- treatments; these treatments were used to guide for different future yield rates. It should be noted that growers do occasionally add N fertilisers to legumes to aid establishment at the beginning of the growing season, especially in mixed pastures.

Testing of N status maker gene expression of the grasses *L. perenne* and *Festulolium* showed were similar patterns to those seen for the NO_3^- treatments in *A. thaliana*. These expression patterns are shown in Figure 3.4 alongside later biomass vegetative yield. The genes *NIR*, *RBCS*, and *NADH-GOGAT* again showed an increase in their expression with increasing NO_3^- concentration, until a peak at ~ 10 mM whereupon expression decreases. This decrease in expression in the higher concentrations also matches with a slight decrease in later yield shown in t ha^{-1}. As with *A. thaliana*, *TIP1* showed a decrease in expression with increasing NO_3^-

concentration. This downregulation pattern of *TIP1* can be useful in conjunction with the other upregulated genes. Conversely, *M. sativa* cultivars did not have patterns of expression that were easily related to NO_3^- supply and subsequent yields, as shown in Figure A3, Appendix A. As mentioned previously, this may be due to N fixation occurring during low supply, it being a nodulating legume. A lack of clear patterns may be due to the narrow range of NO_3^- concentrations tested in the glasshouse, however, testing anymore may not be relevant to the situation in the field. As the data suggests that N status marker genes would be most useful to test in grasses, it was decided that more glasshouse *Festulolium* testing should be carried out with an increased number of NO_3^- treatment concentrations. This was done to obtain more data points within the expression pattern ranges, but also as *Festulolium* is grown across a wide range of UK sites, and frequently in numerous fields on one soil type but with variable nutrient supply. *Festulolium* was used in experiments due to it being a commercially relevant crop, and although other grasses especially *Lolium* or *Fescue* cultivars are interesting to study, the hybrid vigour of *Festulolium* makes it most relevant to the UK forage industry. Fourteen NO_3^- concentrations ranging from 1 – 39 mM were tested, with both 3-week gene expression and 6-week vegetative yield measured as before. The results of this expanded assay are found in Figure 3.5. By increasing the number of concentrations tested it was clear to see that both *NIR* and *NADH-GOGAT* in Figure 5 a) and c) have increased expression until around 21 mM treatment, which matches the drop in later yield. For example, for *NIR* measurements 19, 21, and 23 mM treatments relative expression drops from 4500 % then 1200 % to 250 % respectively, with a change in yield of 7.12 t ha^{-1}, then 7.2 t ha^{-1}, down to 6.3 t ha^{-1} respectively. *RBCS* also decreased expression at the higher ranges shown in Figure 5 b), however this is at 23 mM and the decrease in transcript is more variable with larger error bars at 26 and 29 mM nitrate. This may be because *RBCS* is highly expressed in all plant vegetative tissues, and although soil NO_3^- concentration does affect its expression, the differences are less apparent and more variable at higher supply. As before, *TIP1* shows decreased expression with increasing NO_3^- concentration. The expression patterns found for *NIR*, *NADH-GOGAT*, and

TIP1 under different NO_3^- concentrations in glasshouse conditions were consistent and sufficiently reliable to be used to test field samples.

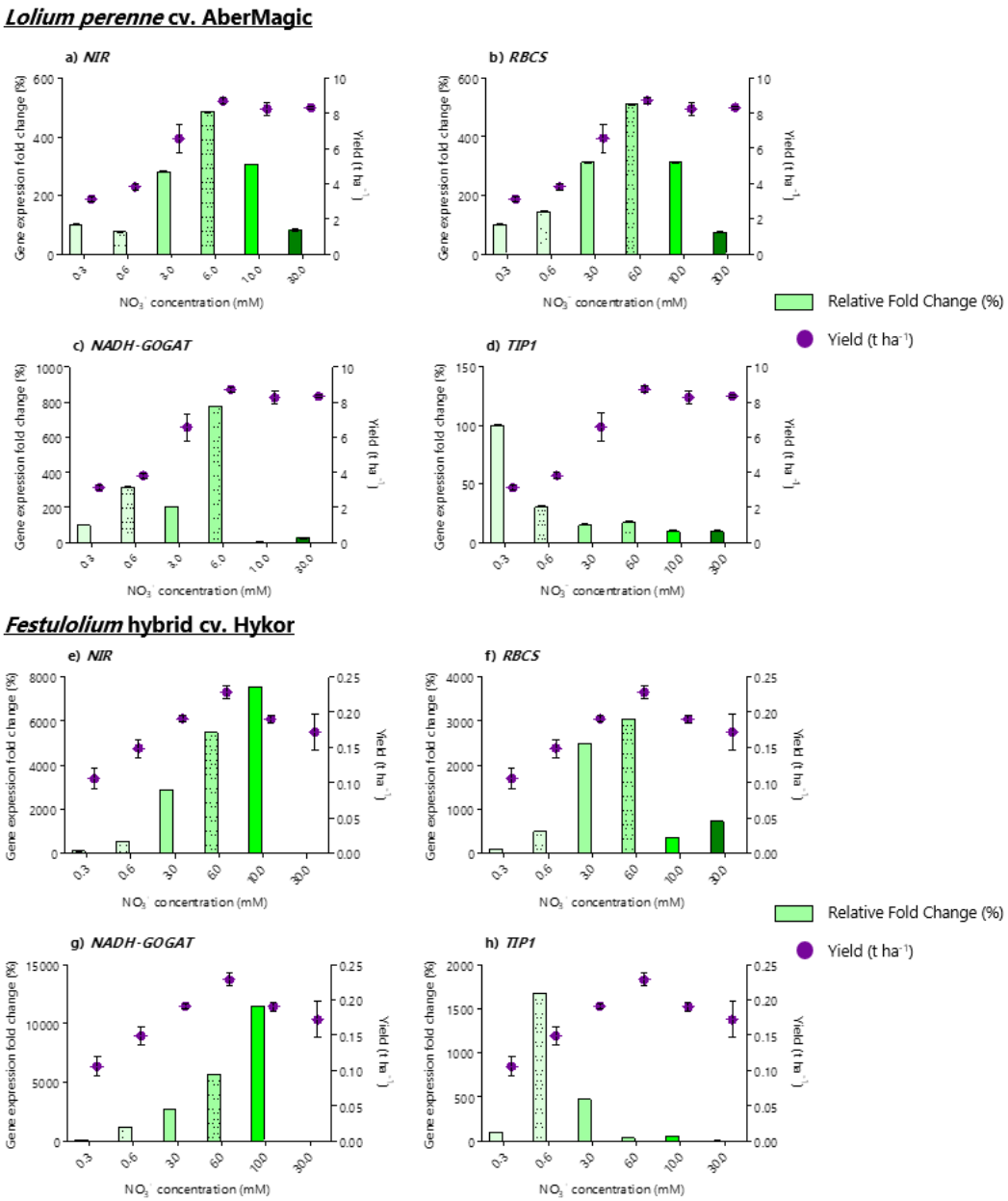


Figure 3.4: Gene expression patterns of N status marker genes in grasses *Lolium perenne* and *Festulolium* hybrid vegetative tissue at three weeks. Expression was calculated as the geometric expression percentage of independent $Ca(NO_3)_2$ and KNO_3 experiments relative to lowest NO_3^- concentration (0.3 mM shown as 100 % in pale green). Bars are denoted for increasing concentration using darkening shades of green, and error bars are standard deviation of expression using two reference genes with 12 biological replicates, in two independent experiments. Expression of genes was shown to vary dramatically in relation to NO_3^- concentration with good agreement between species. Genes tested in grasses were; a) and e) is *NIR*; b) and f) is *RBCS*; c) and g) is *NADH-GOGAT*; d) and h) is *TIP1*.

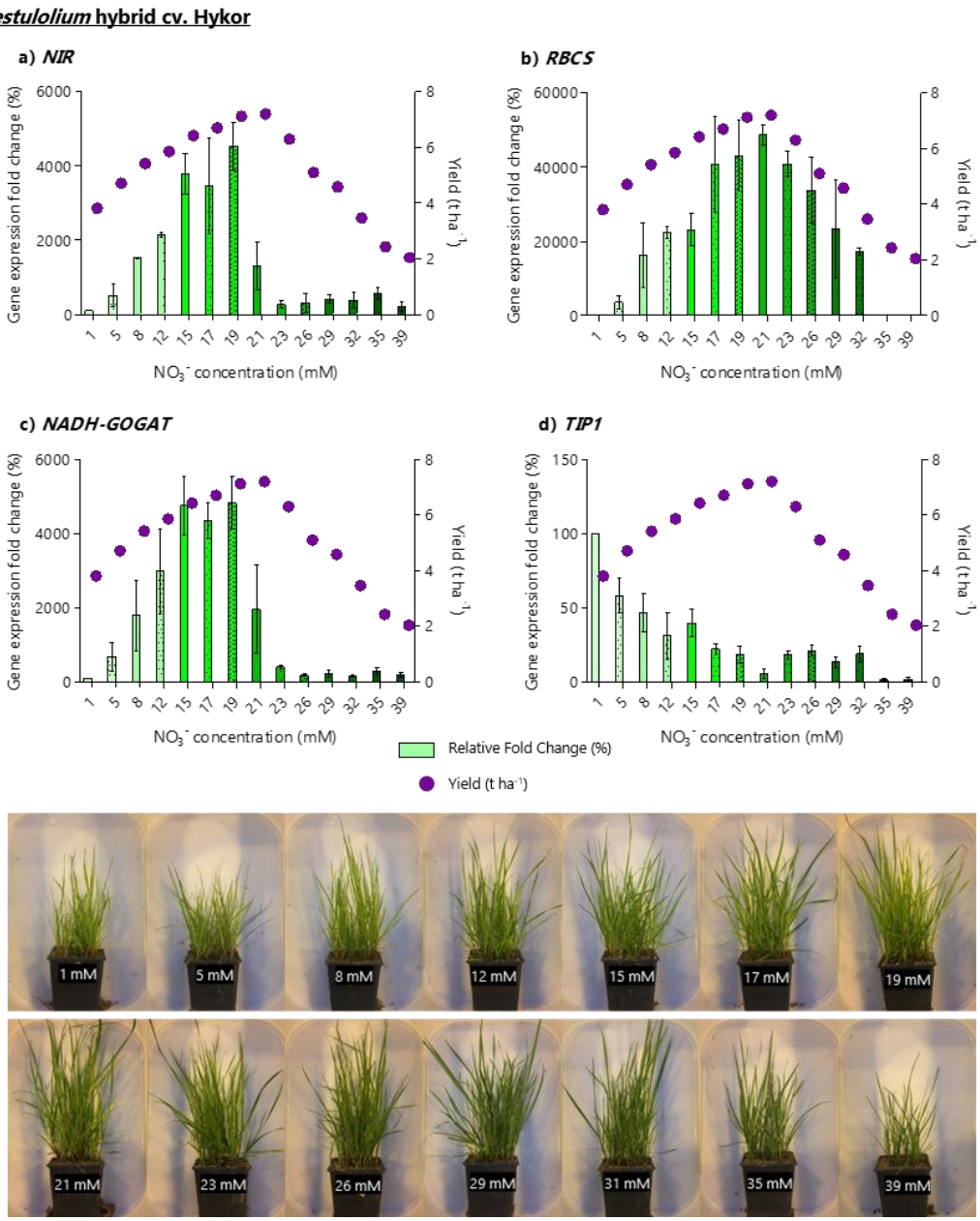


Figure 3.5: Gene expression patterns of N status marker genes in *Festulolium* vegetative tissue at three weeks, with yield photographs.
Expression was calculated as the geometric expression percentage of independent $Ca(NO_3)_2$ and KNO_3 experiments relative to lowest NO_3^- concentration (1 mM shown as 100 % in pale green). Bars are denoted for increasing concentration using darkening shades of green, and error bars are standard deviation of expression using two reference genes with 12 biological replicates, in one independent experiments. Expression pattern of genes varied dramatically in relation to NO_3^- concentration, with later yield also plotted (purple dots). Genes tested were; a) *NIR*; b) *RBCS*; c) *NADH-GOGAT*; d) *TIP1*. Photographs show visually the yield of *Festulolium* effect by different N concentrations at six weeks.

3.2.4 Glasshouse testing of N status marker genes in vegetative tissue could be used to assess field samples using a Gaussian model approach

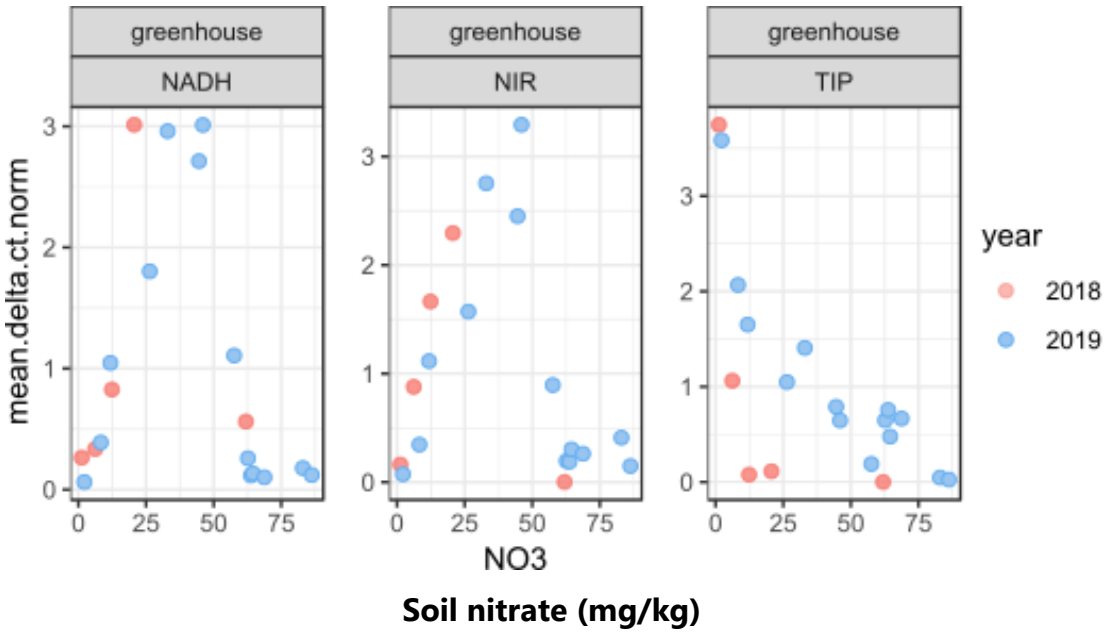
To determine whether the expression of these candidate N status genes was relevant to samples taken from the field a round of preliminary testing was carried out. The aim was to compare the results from the first glasshouse screen of gene expression patterns, in Figure 3.4, with field samples collected in 2018. At the beginning of the growing season, samples of *Festulolium* cv. Hykor were taken from six individual fields at a BAGCD site. Samples were taken in triplicate for measuring gene expression during growth period shortly after fertiliser application, but also for chlorophyll and protein levels. Soil samples were taken at the same time to analyse extractable NO_3^-. Raw gene expression values for *NIR*, *NADH-GOGAT* and *TIP1* of 2018 field samples were calculated in relation to two reference genes. Gaussian process models were fitted to the glasshouse data generated in Figure 3.4 and used to analyse this field expression data. The results of this can be found in Appendix A, Figures A4, A5, A6, and A7.

A second round of field sampling was implemented using crops from the 2019 growing season and with more fields available to sample. Again a Gaussian process model approach was used for this dataset, using the glasshouse measurements from Figure 3.5; these can be found in Appendix A, Figures A8, A9, A10, and A11. As this was also successful, the 2018 and 2019 field datasets were combined and analysed together using a model based on all the glasshouse measurements. The results of this analysis are discussed next, with Figures 3.6, 3.7, 3.8, 3.9 and 3.10.

The glasshouse gene transcript measurements from Figures 3.4 (2018 in red) and 3.5 (2019 in blue) were used to calculate individual normalised gene expression patterns relative to the two reference genes (Figure 3.6). This was called the 'mean.delta.ct.norm' for *NADH-GOGAT* ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') respectively. These were plotted against measured soil NO_3^- concentrations in mg/kg, referred to as 'NO3'. One can see the gene expression patterns as previously discussed; *NADH-GOGAT* and *NIR*

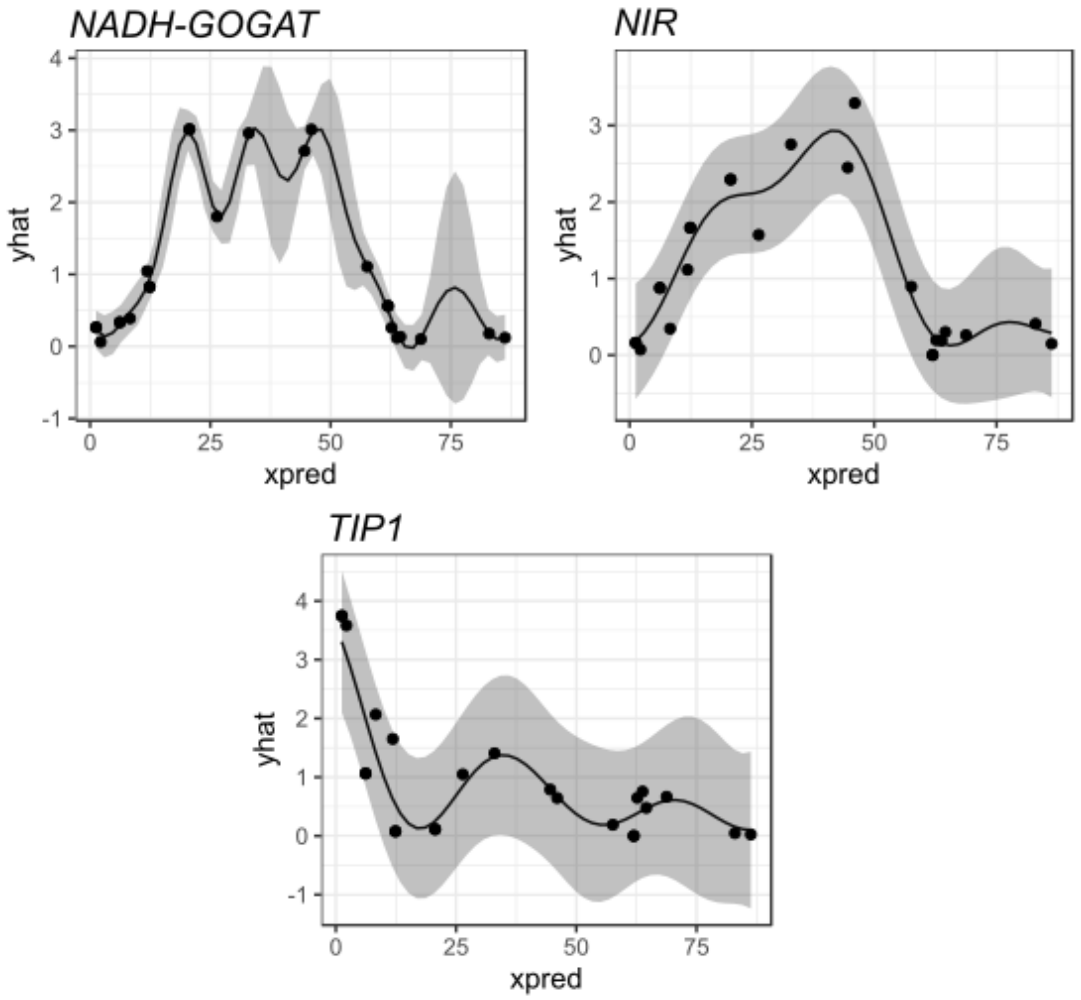
show increases in relative expression with increasing NO_3^- concentrations, until a drop after ~ 50 mg/kg, which is comparable to 19 – 21 mM NO_3^- treatment, and *TIP1* has a steady decrease in expression relative to increasing concentration.

Figure 3.7 shows how a Gaussian process model were fitted to each gene transcript individually. This generates a mathematical expression for the estimated gene expression level ('yhat') as NO_3^- concentration in the soil ('xpred') varies for each gene. y is the mean of all gene expression values for 'mean.delta.ct.norm' as in Figure 3.6. In the case of glasshouse data, 'xpred' is the measured concentration of soil NO_3^-. However when testing field samples, this will be unknown and the model will interpret the raw geometric expression data for each gene to predict the most likely soil NO_3^- concentration, which will be checked against later laboratory analysis.



<u>Figure 3.6:</u> Normalised expression data 'mean.delta.ct.norm' of 2018 and 2019 glasshouse grown *Festulolium*.

NADH-GOGAT ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') normalised expression data was calculated from glasshouse measurements in relation to two reference genes, to generate values for 'mean.delta.ct.norm' against known soil 'NO3'. Original glasshouse data as relative fold change expression is found in Figure 3.4 for '2018' (red), and Figure 3.5 for '2019' (blue).



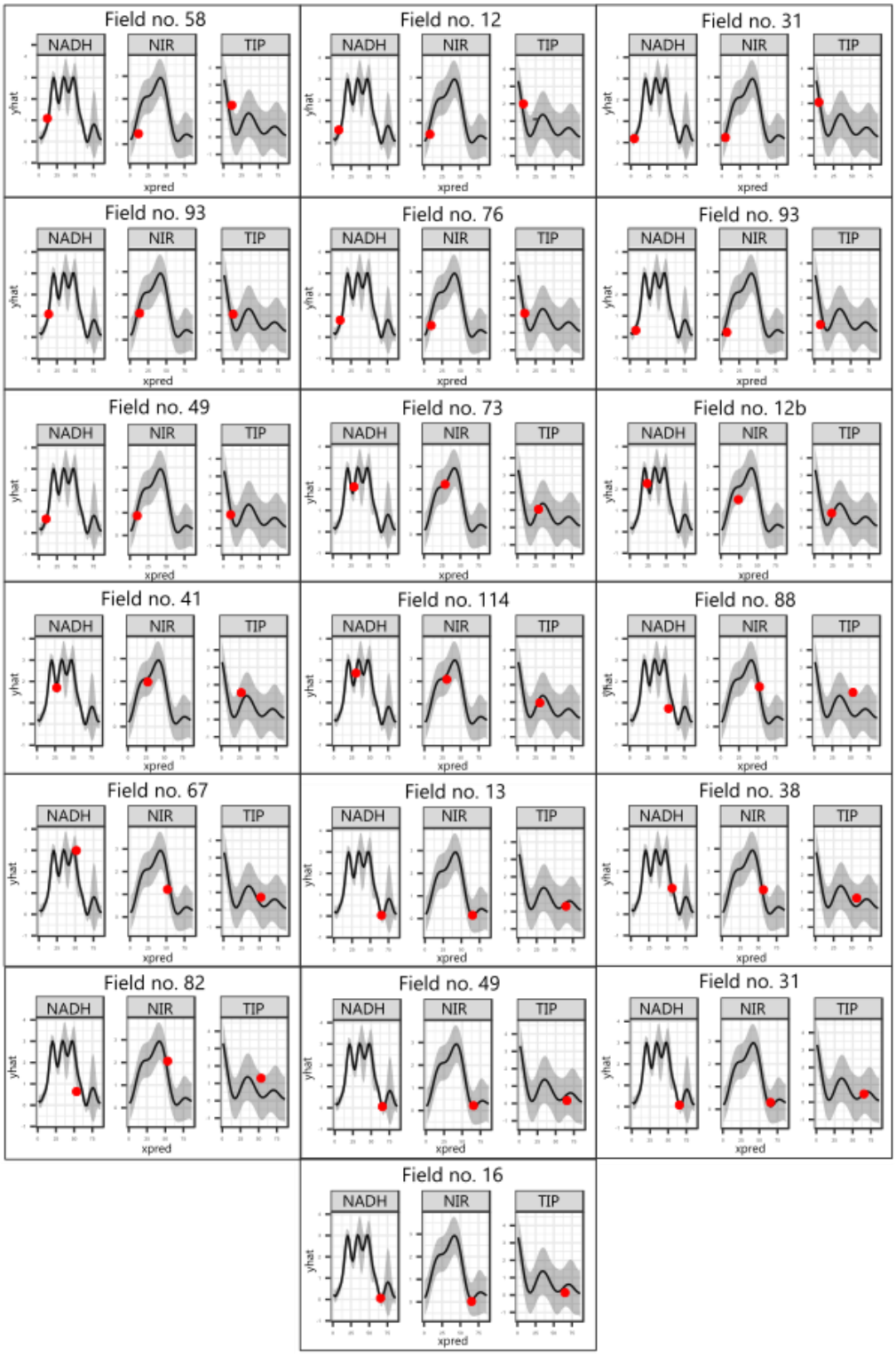
<u>Figure 3.7:</u> Gaussian process models of 2018 and 2019 glasshouse grown Festulolium.

NADH-GOGAT ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') normalised expression data generated from glasshouse data with a Gaussian model applied to individual genes; y-axis ('yhat') shows experimental and predicted values for 'mean.delta.ct.norm' as in above figure; this is required for *NADH-GOGAT* and *NIR* to ensure the model can distinguish the low NO_3^- and high NO_3^- despite the 'mean.delta.ct.norm' being similar in range. The x-axis ('xpred') is the measured soil NO_3^- concentration derived from greenhouse experiments; when field data is analysed with the model it is the 'xpred' which will be deduced from the raw geometric expression data provided. Error region in light grey shows the 95% confidence interval of the model.

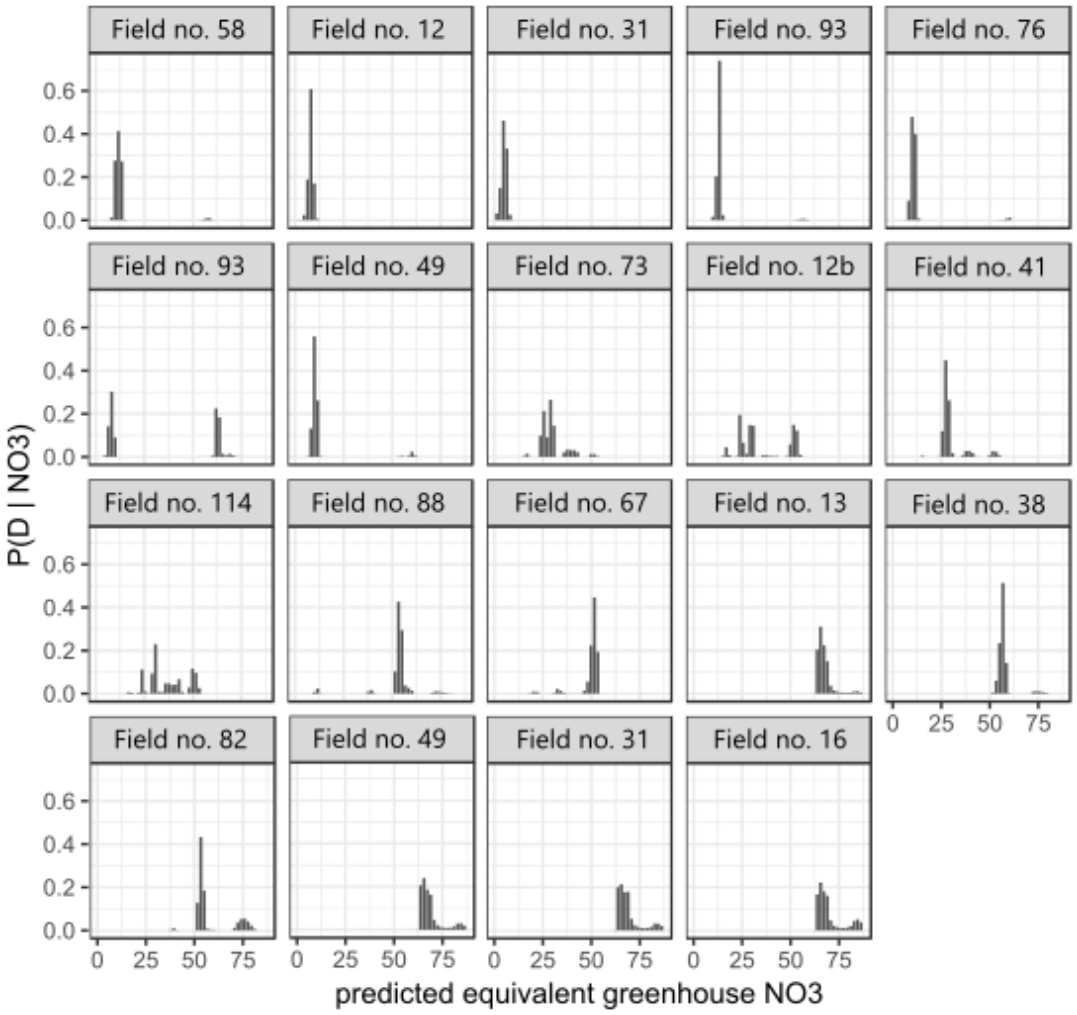
Next, all field geometric expression data was assessed against the Gaussian process models generated in Figure 3.7. Raw expression data was uploaded for each field, and compared against the model predicted expression of *NADH-GOGAT* ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') (Figure 3.8) at each candidate NO_3^- concentration. The value for the normalised gene expression is shown on each graph as a red dot, at the most likely `xpred` value. For example, for Field no. 58 the normalised gene expression, for *NADH-GOGAT* was ~ 1, for *NIR* ~ 0.5, and for *TIP1* ~ 2. This gives a predicted soil NO_3^-, or 'xpred', of ~ 12.5 mg/kg. All fields are placed in order for their 'xpred' value, and so from top left to bottom right the normalised gene expression calculated in the model corresponds to higher predicted soil NO_3^-; Field no. 58 – 76 are low predicted soil NO_3^-, Field no. 12b – 67 are in the middle range, and Field no. 49 – 16 are high predicted soil NO_3^-.

Finally, the analysis of individual gene models shown in Figure 3.8 can be combined to give a single bar chart of each field, provided in Figure 3.9. For each possible NO_3^- concentration (x-axis), this shows the model estimated probability that the gene expression of the three genes arose from a plant grown at this NO_3^- (y-axis), based on the similarity of field gene expression to data generated in the glasshouse. The probability is calculated for each candidate NO_3^- concentration, giving a probability distribution over the estimated NO_3^- for each field.

Therefore the y-value is an indication of the confidence of the model (the higher and thinner the distribution the greater the confidence in predicted soil NO_3^-). For example Field no. 58 shows a relatively tall and thin bar, y ~ 0.4, with an output of x = 7 – 14 mg/kg. This again suggests Field no. 58 has a low soil NO_3^- concentration, also suggested for Field no. 12, 31, 93 and 78. In contrast, Field no. 82, 49, 31, and 16 have lower bars skewed across the upper range of predicted soil NO_3^-, indicating these field have a high concentration above the range for optimal growth found in glasshouse assays, see Figure 3.5. This analysis shows how raw gene expression of three genes measured with plant tissue, relative to two reference genes, can be used determine a value for predicted soil NO_3^-.



<u>Figure 3.8:</u> Analysis of individual gene models for field grown *Festulolium*. Field sample expression data was calculated in relation to two reference genes and the normalised gene expression for each gene calculated. Gene expression at varied NO_3^- was predicted using Gaussian process models fit to glasshouse gene expression data, found in Figure 3.7; 'yhat' is the mean values for 'mean.delta.ct.norm', and x-axis 'xpred' is predicted soil NO_3^-. Red dot shows the gene expression, and maximum probability NO_3^- concentration for each field.

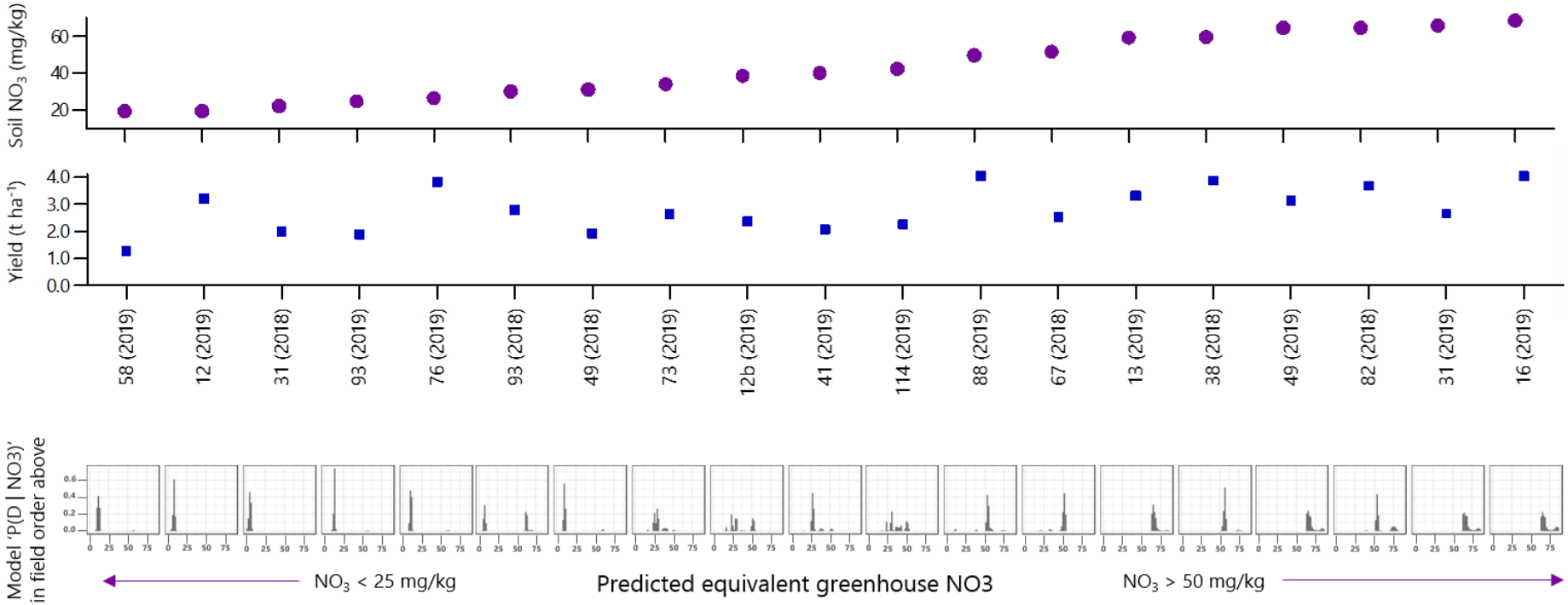


<u>Figure 3.9:</u> Analysis of combined gene models for field grown *Festulolium*. Field sample combined normalised gene expression analysed with glasshouse generated Gaussian model. Here y-axis is 'P(D|NO3)' which is the estimated probability that field gene expression values would be produced at 'xpred' values shown. The x-axis is again 'xpred', here termed 'predicted equivalent greenhouse NO3'. This provides a visual representation of predicted concentrations in the field soil with the higher the 'P(D|NO3)' bar points the more reliable the interpretation of predicted soil NO_3^-. The x-axis shows if the plots are skewed for $x < 25$ this means predicted soil NO_3^- is low, between $x = 25 - 50$ then predicted soil NO_3^- is within an optimal range for yield, and if $x < 50$ then concentrations are deemed higher than required for efficient growth, based on glasshouse yield observations.

3.2.5 N status marker genes can be comparable to current conventional testing, providing information of both crop and soil status

As Figure 3.9 shows how gene expression of three N status marker genes can be used to predict soil NO_3^-, it is necessary to compare these to actual soil NO_3^- concentration measurements. Moreover, if predicted soil NO_3^- matches actual measurements from soil extractions sampled on the same day as tissue for expression, it will be interesting to see if there is a later correlation to yield of each field measured in the subsequent weeks. Figure 3.10 presents three datasets for field samples. At the bottom of the figure, discrete field 'P(D|NO3)' against 'predicted equivalent greenhouse NO3' is shown from Figure 3.9, in order from low predicted soil NO_3^- to high, indicated with text and purple arrows. Above these are graphs of actual measurements for extractable soil NO_3^- as mg/kg (purple dots), and later yield as t ha^{-1} (blue squares).

Essentially, if one compares the predicted model generated bar charts for soil NO_3^- against the actual extractable soil NO_3^- there is a high rate of agreement in the measurements; the model correlates with actual measurements for soil NO_3^-. Moreover, in Appendix A, Figure A12 shows the actual extractable soil NO_3^- correlated to predicted soil NO_3^- based on the highest 'P(D|NO3)' bar point, showing an $R^2 = 0.92$.



<u>Figure 3.10:</u> Extractable soil NO_3^- and *Festulolium* yield for fields from 2018 and 2019, with comparison to combined gene models generated with glasshouse data.

Extractable soil NO_3^- as mg/kg sampled at time of expression sampling is shown in top graph as purple circles ('Soil NO_3'), and crop yield in t ha^{-1} measured ~ 3 weeks after sampling is shown below in blue squares ('Yield'). At the bottom, 'P(D|NO3)' is the estimated probability distribution across predicted greenhouse equivalent NO_3^- concentration. Plots provided from Figure 3.9 to compare to values for 'Soil NO_3' and 'Yield'.

3.3 DISCUSSION

3.3.1 Testing N status candidate genes provides more information of how NO_3^- environment can affect NUE associated gene expression

This chapter showed that the candidate N status marker genes in *A. thaliana* have clear patterns of expression between various NO_3^- treatments (Figure 3.1). This has confirmed the expression pattern reported in the literature for some of these genes under different NO_3^- concentrations in an independent study. For example, both *NIR* and *NADH-GOGAT* showed similar expression patterns in *L. perenne* and *Festulolium* to those described in *A. thaliana* [377; 378; 134; 133].

The discovery that *TIP*s were downregulated pattern was unexpected. As some *TIPs* are vacuolar markers for protein storage vacuoles (PSVs) and are well documented as mediators of water and ammonia transport [316; 379; 380], it was speculated that they would increase in expression following higher NO_3^- supply levels due to increased PSV numbers in the tissue. However as the data does not support the prior assumption that PSV numbers will increase with higher nitrate supply it is possible that although the *TIP*s are important in vacuolar transport, they may be important for not just PSVs but also LVs [320; 381; 382]. Therefore, turnover of substrates depending on whether the vegetative tissue tested is source or sink. Nevertheless, it is unclear how specific individual TIPs function, with TIP1s and TIP2s both being association with large LVs and PSVs [319; 383], with cellular specificity rather than tissue. More exploration of TIPs in monocots could provide answers to these specificity questions, as *A. thaliana* has been the primary focus of their investigation so far.

In addition to this, the vegetative tissue assessed in this study is only three weeks post germination. This means there may be high differences between expression in young sink leaves and older source leaves. This

would be true for *A. thaliana* studies, but as a single grass leaf has both old and young tissue present on sampling this could be worth further investigation for protein storage in monocots, as well as general water movement. This work further supports the idea that more investigation of TIP function is needed. Areas of TIP function to be addressed before assumptions can be made regarding their specificity across plant tissues include whether TIPs are localised to PSVs or LVs, and how strictly localised they are in certain tissues of different ages.

The development of N status marker genes in forage crops provides tools for future investigations. Firstly, the design and use of primers for *L. perenne*, *Festulolium* and *M. sativa* could be used in gene expression studies testing parameters of fertiliser application, rate, and regimes. These primers are compatible in both glasshouse and field studies, and so could be an excellent tool for breeders in targeted NUE programmes. This was not true for *M. sativa,* where N-fixation by the legume may be complicate the picture. To test this idea, it would be interesting to check the pattern for expression of my suite of marker genes when the legume is grown in aseptic culture when N fixation is not possible and increasing NO_3^- is supplied.

The results of the preliminary round of field testing were positive, especially for using plant gene expression data to infer soil NO_3^- status without need for soil extraction. This approach was successful in transferring a technique established in the glasshouse to the field. Success could be measured statistically by how well the model fitted the combined field sample data.

This means that the sampling of vegetative tissue at three points of a field and analysing its expression of only three N status marker genes is enough to accurately determine its actual soil NO_3^- without need for laborious soil extraction. This information could be used to aid management practices, for example apply fertiliser to a low concentration field as the plant is growing to increase yields before harvest.

In addition, it is possible to also compare the predicted model bar charts for soil NO_3^- against the actual future yield of each field in t ha^{-1}. Although this does not correlate as strongly as actual extractable soil NO_3^-, one can

see a general trend where the higher the predicted soil NO_3^- then the higher the subsequent later yield of the field (see Figure 3.10). This is not surprising, as a field with a better N status should be able to support higher yield of vegetative crop. However the correlation is not strong enough to support its own model with outliers in yield found including Field no. 12 (2019), 49 (2018), 88 (2019). This is likely due to more convoluting factors affecting yield in the field when compared to glasshouse measurements. Moreover, no correlation for extracted vegetative chlorophyll or protein could be found with yield, shown in Appendix A Figure A13, suggesting more factors are at play in determining yield variations. There also seems to be no significant drop in yield at the higher soil NO_3^- concentrations found in glasshouse assays.

3.3.2 Fields are variable for both soil N and crop yield

The high spatial variability of soil N is known to affect its biological communities [384-390]. This heterogeneity is shown in Figure 3.10 where both extractable soil NO_3^- and *Festulolium* yields have large ranges of measurements; soil NO_3^- between fields ranged from 19.44 - 68.5 mg/kg based on three samples per field, and crop yield ranged from 1.28 - 4.03 t ha^{-1}. These large ranges are found on a relatively small farm area, see Chapter 2, Figures 2.1 and 2.2 for fields sampled, despite the fields having similar soil types and texture, and farming management practices, including fertiliser application rates were parallel.

The variation found in soil N levels is likely to be caused by the natural heterogeneity of land even at small scale found especially in temperate regions such as the UK [391-397]. Such variation makes the low frequency of current sample practices for UK dried forage producers (often just 3 - 5 soil cores per field for BAGCD members [398]), inadequate for grower needs where precision farming techniques are becoming more desirable.

The high variation in field yields measured in this study shows how forage cropland and grassland are affected not only by differences on soil nutrient levels especially N heterogeneity [399; 391; 400]; soil NO_3^- and field yield have different coefficients of variation at 40.13 and 29.27 respectively. It

also indicates how other factors are likely to influence forage yield, including soil acidity [401; 104; 402; 403], water availability [404; 405; 205], and disease susceptibility [406-409]. Interestingly correlation of vegetative yield with chlorophyll and protein concentrations found in this study may support the usefulness of fluorescence with drones and SPAD meters for decision support, but the precision of lab-based measurements may not be matched by these field monitoring methods. Moreover, as such factors are interconnected especially when the complication of climate change is included. Many studies are beginning to explore how to model these factors integrating their effects on yield [410; 411].

3.3.3 Refining N status marker gene suite with model provides promising developments for grass research

Despite similarities in cultivation, the measurements for soil N and yield drastically highlight the need for growers to be able to assess individual fields when required, and not be trapped with conventional methods which are costly and time-consuming [376], even if they are necessary to comply with regulatory standards [412; 413; 79; 414; 415]. Moreover as heterogeneity within individual fields is so large methods that allow for rapid sampling at a few points across an area provides a more accurate representation of field soil status.

In this study it has been shown how a relatively non-invasive technique measuring a small amount of plant vegetative tissue from three field points can assess soil N levels rapidly across many fields. Although requiring scientific skill to conduct, the measurements for soil NO_3^- have been shown to be robust using gene expression models built using glasshouse measurements. This data could be improved through more testing, particularly in the field. Although some training would be required, the current testing of soil extracts especially using soil suction cups has limited precision and scope. Crop growers including BAGCD members are increasingly wanting to have more exact tools for assessing their crops and soil, so such techniques are gaining more practical need. Such techniques are also incredibly applicable to breeding programmes who already have a

wealth of scientific training. Moreover, there is continued development showing use of handheld PCR kits in the field [416-419] which are easy to use for a small number of parameters; such a technique may be utilisable for the current suite of three N status marker genes alongside two reference genes. Of course the downside of using the suite of marker genes in any crop is that when the PCR method detects a sub-optimal soil nitrate supply this is already too late for the crop and the maximal yield will not be achieved. But this criticism is valid for any methods that detect a response in the plant.

It would also be interesting to see how other cultivars vary in their marker gene expression related to soil N levels. Variation could be seen in different *Festulolium* cultivars due to their breeding history [420; 2] as can be seen in Figure 3.4 with its slight N specificity differences to *L. perenne*. This suite of genes may potentially be important markers for NUE in forage grasses that can be used in breeding programmes.

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Chapter 4: Soil sensors can detect N profile changes under forage crops with different management regimes

4.1 INTRODUCTION

4.1.1 Nitrogen fertiliser production for crops is demanding

The Haber-Bosch process for fixing free nitrogen (N_2) from the air and converting it into ammonium (NH_4) is arguably the most dramatic invention in modern human history. Along with the breeding of dwarf varieties of the main cereals and developments in herbicide and pesticide production, this fixation process was part of the Green Revolution which allowed crop production outputs to increase by a dramatic four-fold [421]. Due to its contribution to human history with a huge increase in crop production causing an increase in human population, the technique has been termed the 'detonator of the population explosion' [422].

Fritz Haber and Carl Bosch's process was developed from Haber's 1908 "synthesis of ammonia from its elements" patent [423]. The work won each of them a Nobel Prize. More notable than its scientific importance in the field of chemistry, ammonium fertiliser production changed the impact of the late-industrial age globally by feeding 50 % of the world population through increased grain yields [424]. The process converts atmospheric N_2 to NH_4 under high temperature and pressure and in the presence of a catalyst, typically iron-based. It should be noted that despite this incredible development in fertiliser production, the Haber-Bosch technique was originally developed in Germany with ammunitions as its motivation, and ironically Haber's work can also be linked to the deaths of hundreds of thousands of people [425; 426].

Anthropogenic activities fix 210 Tg N $year^{-1}$ [427] with total N input for food production globally at 171 Tg N $year^{-1}$, half of which is accounted for by the animal products industry [428]. The process is resource and financially exhaustive; the practice uses 3 - 5 % of the world's natural gas, and 1 - 2 % of its energy [429]. Recent studies have aimed at increasing efficiency of N fixation by manipulating the Haber-Bosch technique with specialised catalysts and plasma electrode fields [430-435], but still global N demand continues to increase. This is also being stretched by its

potential use in transport fuel and water space heating with further technological advances on the process multiplying [436]. Emissions of N from fertiliser are estimated to be at around 10 % of global outputs [437], with agricultural emissions from animals and manure being responsible for the bulk of this loss [438]. Emissions had previously been shown to be highest in Asia, predominantly China and India, and Europe [439; 9]. However. other areas of the world are now catching up due to increases in synthetic fertiliser use intensity [393; 427]. Western Europe including the UK uses 7.3 % of the world's synthetic N consumption [440], which is disproportionate with its landmass size.

This resource consumption and subsequent emissions have dramatic consequences for the world. The vast energy required for N fertiliser production puts a strain on energy demands, and using a huge supply of natural gas directly contributes to our unsustainable dependence on fossil fuels. This is directly beginning to influence governmental policies and is continuously monitored in countries including the UK [77; 78; 394; 441; 414; 81; 82; 442; 83; 415]. There is a political and financial aspect to using fertiliser more efficiently.

There are serious environmental concerns regarding N fertiliser use. Leaching of N leads to eutrophication of water supplies where algal blooms in rivers and lakes limits sunlight, space and oxygen for aquatic species and therefore leads to high death rates in these ecosystems [443; 444]. Such leaching can also contaminate human drinking water especially in ground water supplies. Forage grass and legume crops have long been thought to have high rates of leaching to the environment [413]. This problem can be exacerbated when they are grown in sandy soils, the choice for many forage crops grown on BAGCD sites. Some estimates are that 60 % of applied N is lost through leaching, run-off, denitrification and consumption by microbial populations. Furthermore areas termed Nitrate Vulnerable Zones (NVZs) make up approximately 58 % of the land in England [77; 414; 81; 82; 445]. These areas include farms used by forage growers such as the BAGCD.

There are also arguments for an impact of N-fixation on human health issues including infantile methemoglobinemia (blue baby syndrome) and

the formation of N-nitroso compounds that are potential carcinogens, formed by commensal bacteria from nitrate, although there is much need for further studies into such effects [446]. The impact of drinking-water being contaminated with leached N on human health may results in decreased life expectancy [446-448]. Assessment studies for the social cost of nitrogen (SCN) have been implemented [445]. Finally there is growing evidence that N emissions, which are 0.5 - 1 % of production inputs [440; 449; 450], may contribute to climate change, as a by-product of fertilizer production [451-457]. Nitrogen oxides (N_2O and NO) may be the single largest contributor to atmospheric greenhouse gases [458; 456; 459; 460]. Ammonia emissions are most relevant to grasslands and have been investigated in the UK [461; 462; 458]. These emissions can be produced as a by-product of N fertiliser use through microbial breakdown.

Increases in global demand for protein are estimated as 110 % ± 7 % [463]. This will undoubtedly exacerbate the current problems with N fertiliser use as an increase in food production is required [464]. The rate of N fertiliser use in the UK compared to global consumption has recently declined slightly by almost 1 %, although consumption still stands at a forecasted 7.13 % for the next decade [449]. The future use of fertiliser is of increasing concern for crop growers worldwide. Environmental concern over N fertiliser use by farmers has been hotly debated due to the potential adverse effects of leaching into ecosystems compared with the economic advantage for production. Moreover, there is growing pressure to impose legislation and regulations on farmers due to these detrimental effects. Negative associations between fertiliser use and crop production are increasingly recognised by the public.

Despite synthetic N fertiliser use being a major factor in the global population boom, these negative consequences have caused its use to be deemed ‘too much of a good thing’ [465]. A ‘greener revolution’ is required [466]. This revolution should be directed towards increasing the efficiency with which crops use N fertiliser and decreasing as many adverse effects as possible. One approach is to study N fertiliser supply changes in soils, particularly under grass and forage crops.

4.1.2 Studies have estimated N changes in grassland and forage cropland

Plants are able to take up a variety of N substrates principally in the forms of nitrate and ammonium, but also amino acids and other organic N compounds [266]. Deficiency of N limits crop production by restricting protein assembly, affecting primary and secondary metabolism. This is particularly relevant for the high N demand of the leaf photosynthetic apparatus where deficiency impacts on all growth parameters [75]. Leafy crops including forage grasses and leguminous herbs have a slightly higher N content than cereal grain crops, and require more N for optimal production [74]. Soluble nitrate (NO_3^-) in the soil is the most abundant source of N readily available to the crop [467; 72; 468]. Many forage grasses, such as ryegrass, will preferentially uptake NO_3^- as N source particularly at high fertiliser rates [469; 470]. Although usually above 1 mM, the concentration of NO_3^- in plots can vary between areas metres from one another [399; 391; 392; 400; 393]. Nitrate has been shown to have a particularly high degree of variability between samples [471], with a high potential to leach through soil [472-475]. Some estimates calculated that 60 % of applied N is lost through leaching, run-off, denitrification and consumption by microbial populations [476; 477]. There is a lack of research into leaching when grassland is temporary and intensive for high production, and more research is required with a focus on different soil types and management practices [118]. Moreover, N leaching may vary depending on the crop grown, due to differences in the root structure of forage crops [478].

Long-term experiments into N fertiliser leaching between soil plots have been conducted such as Rothamsted's Park Grass Continuous Hay Experiment, which began in 1856 [479; 104; 480]. One prominent study was a 19-year long assessment of leaching using ^{15}N labelled fertiliser, which found leaching rate of $^{15}NH_4$ and $^{15}NO_3$ to be approximately 13.9 % and 21.9 % of the total N fertiliser application respectively [481]. However, many growers choose to cultivate mixed crops of grasses and/or legumes so the degree of leaching may vary. Other large scale evaluations of how

N application in agriculture affects the surrounding ecosystem have been undertaken. A decrease in grassland plant diversity has been shown following an application of just 2 kg N ha^{-1} $year^{-1}$ [482]. Moreover, studies have shown leaching of inorganic N was lower for grassland than that for other arable crops including cereals [483; 484].

One key reason for lower leaching in grasslands is that the N applied is routinely removed as the plant uptakes the required amount, then removed from the field by either grazing or frequent cutting [485; 486]. Although it is known in *Lolium* that defoliation of aboveground vegetative tissue can halt the uptake of NO_3^- in hydroponics, which could therefore increase leaching of N fertiliser [119], it is difficult to compare leaching rates in permanent grasslands with cultivated forage crops. More research is needed to compare how specific management practices can influence leaching. For dried forage grass production, research is needed on N uptake in newly seeded species, cutting effects on leaching especially in root response and intercropping with leguminous forages.

By understanding the N cycle and being able to measure NO_3^- levels in the soil one can test how well crops utilise fertiliser. This is of particular interest in forage crops, as these areas of farming are vast with high fertiliser inputs and wide-ranging across many species.

4.1.3 Current testing methods of soil N are complicated to use

At present the measuring of soil N has been argued as unreliable, inaccurate and inconsistent across land and within the industry [376]. Farmers and agronomists aim to make detailed plans for their fertiliser applications and crop rotations, that depend on the local weather to extrapolate a baseline field soil N content. This estimate depends on soil sampling, with soil cores sent to independent laboratories for analysis of ammonium, nitrate and nitrite. This convention has existed for many decades [487-490], and includes calculations for soil nitrogen status [491]. Analysis is performed using KCl soil extracted samples and these are calibrated to known standards with chromatography and colorimetric

tests such as the Griess–Ilosvay diazo-coupling reaction [492; 493]. These measurements are converted to quantify the concentration per hectare. Although the procedures have been used for decades, there are notable problems in their uses. There is no standardised protocol for collecting soil samples and their transport, or how these are stored. Net nitrogen mineralization in soils is affected by abiotic conditions [494], including pH [495; 496], temperature [495; 497-500], moisture [497; 501; 500; 502], and soil texture and chemistry [503; 497; 504; 505; 496]. Therefore, the time taken between collecting a sample and laboratory analysis, including the storage during transport may significantly affect the final extrapolated measurement. Other techniques have tried to improve the precision of readings by removing interference from other ions [506; 507] including the deployment of specific test strips [499], or by optimising extraction and colorimetric buffers to accurately reflect soil concentrations [508-510; 496; 493]. Innovations with plant spectroscopy methods can be used in conjunction with these soil analysis to provide a description of the field environment [511].

Porous ceramic cups or soil lysimeter methods can be used to sample N in soil water on farms, with pH indicators used as a proxy for soil nutrient stoichiometry including N levels [512]. The soil water samples undergo the same chemical analysis as soil samples above and is therefore open to the same criticisms. This requires a large number of samples just for one field, to get a good representative amount for the site. The cups can provide large variation in individual measurements from a single cup sample [513]. Moreover, there is no consensus on when soil samples should be taken, after harvest in the winter or in the spring and how long they should be stored before analysis [376]. The N levels in the soil temporarily vary across a season and spatially across depths [384; 391; 400; 471; 514-516; 388; 389]. Methods such as ceramic cup sampling or soil extractions are labour intensive, with a cost impact for each sample. As reviewed by Shaw *et al*., (2016), current methods of testing are labour-intensive, expensive, and not conducive to the aims of the modern drive for precision agriculture [517]. In addition, there is limited sharing of data in the UK across different farmlands making extrapolations of soil N availability to crops difficult.

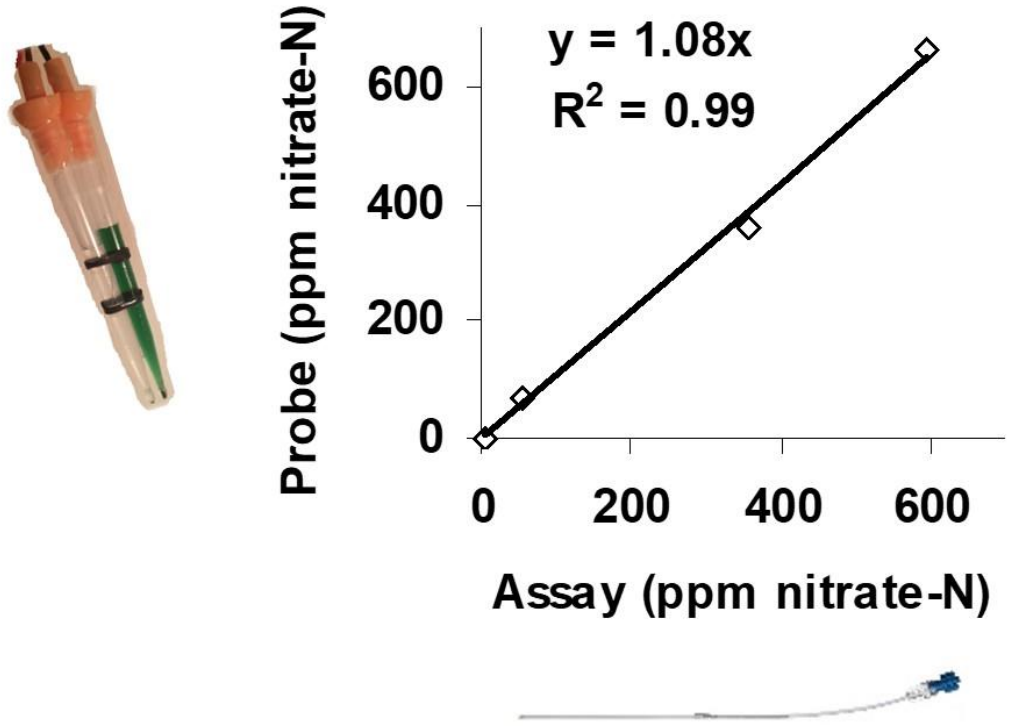
One method is the use of ion-selective sensors such as those used by Shaw *et al*., (2016) to measure *in-situ* real time NO_3^- levels through networks as an alternative to conventional sampling methods described above. Such ion-selective sensors have been developed in the Miller/Sanders laboratory [155] based on earlier work using ion-selective microelectrodes [518; 156; 519]. These sensors can report in real-time the free NO_3^- in the soil water across different soil types and can be calibrated with standard NO_3^- concentrations. It is also possible to use these sensors at different depths to get a measurement of N across a soil profile. Limitations to their use are the design of economically viable sensors and farmer-friendly interfaces to access and store their outputs.

4.1.4 Ion-selective nitrate sensors may be deployed in trials to assess soil concentrations

Sensors which can be used to detect NO_3^- in soil were developed in the laboratory from previous work measuring the concentrations in plant cells. These NO_3^--selective sensors can be made cheaply and quickly, see Figure B1 in Appendix B for details of routine production in the laboratory. These sensors have been tested against the conventional soil testing detailed in Section 4.1.3. The good agreement between sensor readings and conventional testing can be found in Figure 4.1, where an R^2 of 0.99 was observed between the two measurement types.

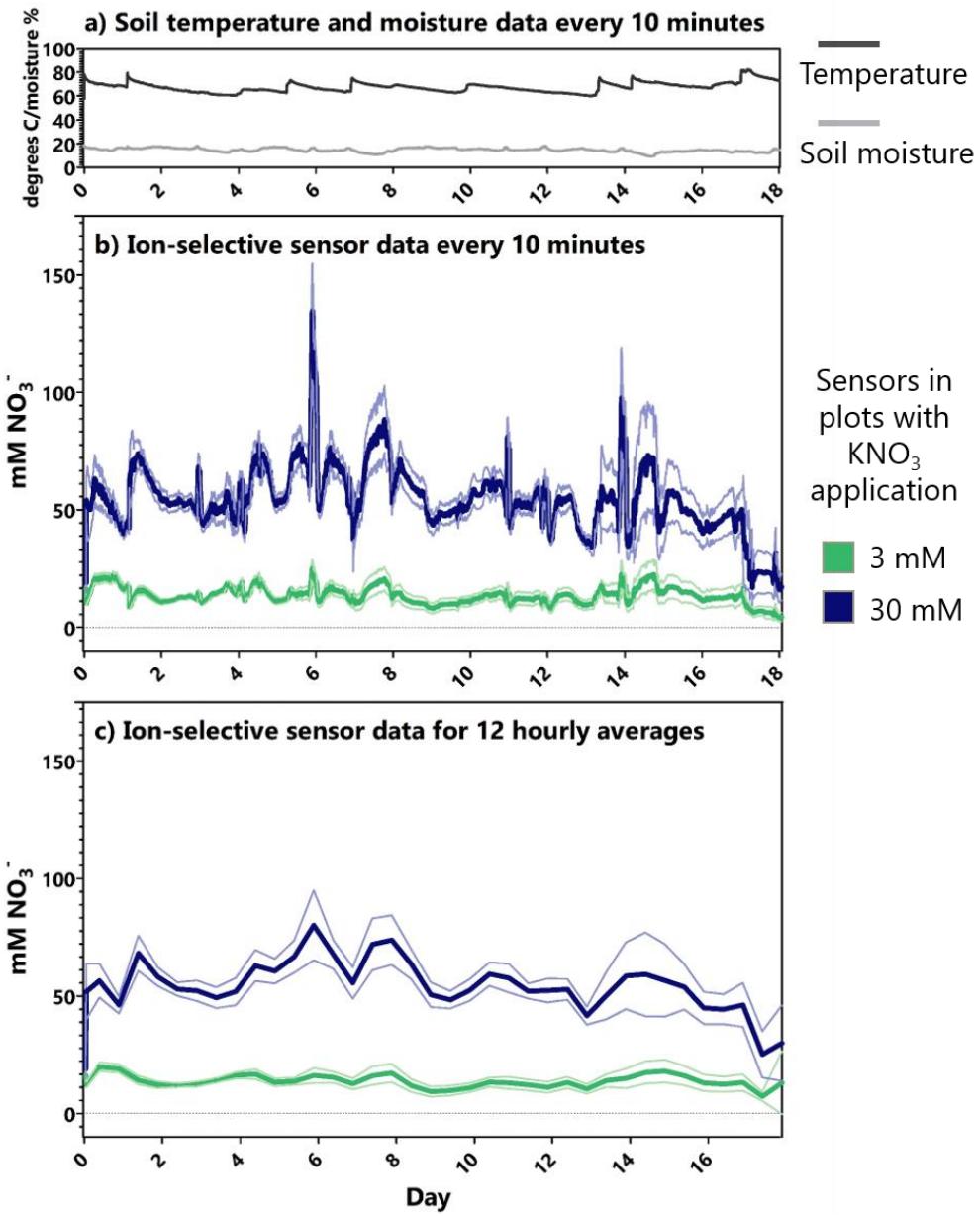
Figure 4.2 below is an example of NO_3^--selective sensor deployment in the field (Figures 4.2b and 4.2c), alongside standard soil temperature and moisture measurements (see Figure 4.2a). In this field example, plots were treated with either high or low NO_3^- solutions of 3 or 30 mM KNO_3 at 0 days. One can see the concentration of NO_3^- recorded by sensors increases upon application in the 30 mM treatments, indicated by the blue plots for both 10 minute and 12 hour average data, increasing from ~ 18 mM to ~ 50 mM NO_3^-. The data sampling frequency was every 10 minutes, but the outputs were also plotted to show 12 h mean values. The trends in the data are very similar and so all future data plots used a 12 h sampling average. No significant change was shown in 3 mM plots, as NO_3^-

remains between ~ 10 – 20 mM. Such data shows real-time changes in the soil water NO_3^- concentrations in the field and may be correlated with biomass yields, protein, and chlorophyll levels of forage crops.



<u>Figure 4.1:</u> NO_3^--selective sensors (Probe) correlated to conventional soil nitrate extraction analysis methods (Assay).

Soil at four known nitrate concentrations was measured for nitrate-N as ppm by both NO_3^--selective sensors and through conventional spectrophotometric assay. Nitrate-N ppm are the units commonly used by soil scientists, the sensors can be calibrated to give outputs in mM too. The above graph shows the strong agreement between measurements, with $R^2 = 0.99$. Graph provided by plotting a scatter plot of each type of data values and fitting a linear regression.



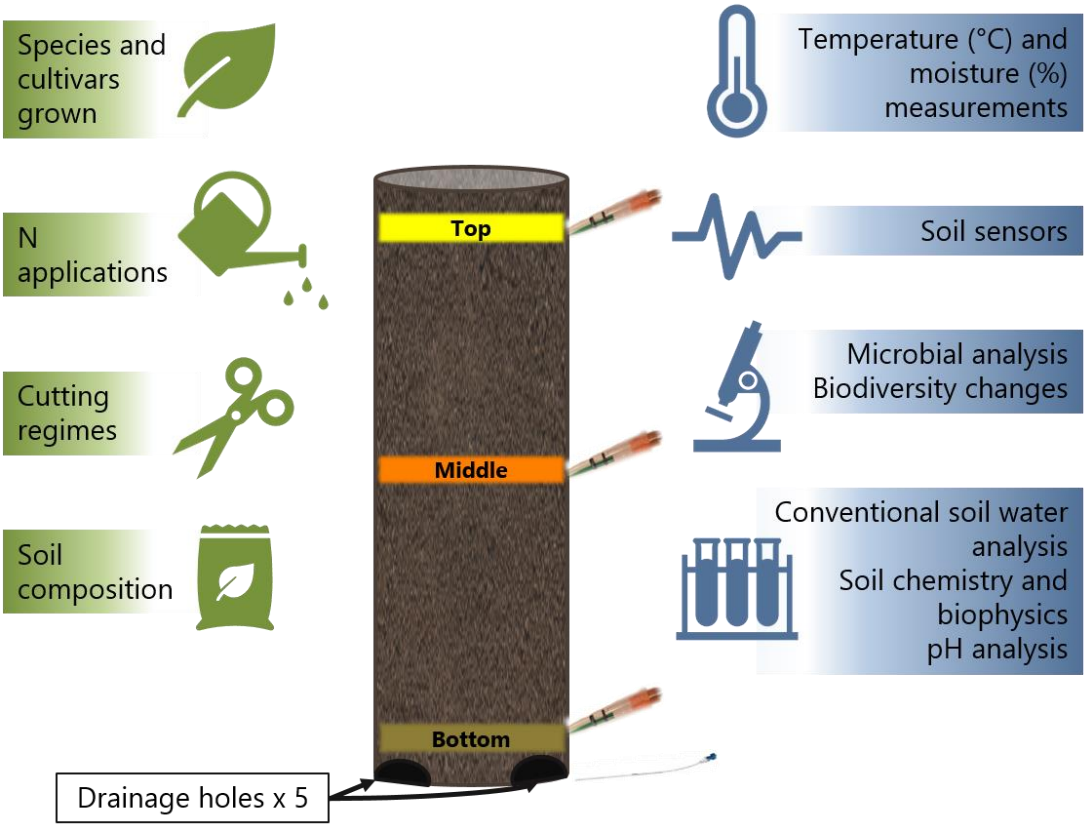
<u>Figure 4.2:</u> Field trial example of NO_3^--selective sensors in plots treated with different potassium nitrate concentrations.

NO_3^--selective sensors were constructed in the laboratory and deployed in the field in two plots at the John Innes Centre, Norwich to a depth of ~ 10 cm. On day 0 plots were treated with either 3 mM (green plots) or 30 mM (blue plots) KNO_3 and sensors prepared as in Section 2.2.3, data-logging along with Delta-T SM300 soil moisture and temperature sensors (Delta-T Devices Ltd., Cambridge, UK). Soil temperature and moisture are shown in a), as dark grey and light grey lines respectively. Ion-selective sensor data is shown for each plot, an average of four sensors with data output logging every 10 minutes in b). A 12 hour average is shown in c). For both b) and c) the errors are shown with lighter bars and are the standard errors of the means.

Although field trials provide the most accurate data for N fertiliser changes in grassland soil, the testing of NO_3^- treatment effects on plant growth and soil profile leaching analysis was moved to column experiments in glasshouses. Deploying many sensors at various depths in the field is technically difficult, especially for measurements below 60 cm in the soil profile. Under these deep conditions the weight of the soil in the field can crush the plastic tips used to make the sensors. Soil column experiments are routinely used in plant and soil science. They have been used to monitor leaching of other minerals including sodium, calcium and magnesium [520]. They are particularly useful for root imaging studies which use x-ray technology to monitor changes in root architecture based on water and nutrient conditions [521]. They can also be used to assess how plants root systems interact with one another [522] and test cultivar phenotypes [523]. Although they are still an artificial system when compared to field measurements, they are superior to artificial media based or hydroponic systems when studying root nutrient uptake. Hydroponic experiments require aeration systems, with management of either sterile or artificial microbial populations. Hydroponic and gel-based media systems lack the influence that soil structure has on root growth and nutrient uptake [524]. Therefore, column experiments are a more realistic model system and useful for studying soil profile changes under forage crops. Column experiments can be useful for replicating soil environments, recreating forage grower management practices with more technical ease than field trials. For example, one can study quite efficiently how two different fertiliser regimes behave in the soil profile, with the confounding influence of cultivar root architectural differences monitored directly. Figure 4.3 illustrates some parameters which can be easily varied in column experiments, as well as the types of measurements which can be undertaken. The dimensions of the columns make them technically manageable (e.g. weight) and forage plants can be sown at industry rates with adequate room for root growth.

As stand-alone systems they can have sensors inserted at different depths, see Figure 4.3, without the problems associated with field use. Soil water can also be taken from the bottom drainage holes using lysimeters to check for leached NO_3^-, using the conventional testing discussed in Section 4.1.3.

Experimental replicates can be carried out with practical ease within a feasible timeframe, providing meaningful data more representative of field phenomena than hydroponic or gel-based media can provide.



<u>Figure 4.3:</u> Schematic of column set-up for NO_3^--selective sensor experiments.

Columns have dimensions of height = 50 cm and inside diameter = 15.4 cm, see Section 2.2.2 for details. Possible experimental regime changes are marked in green, with the different assays possible shown in blue. Holes are available at three different levels for placement of the NO_3^--selective sensors (shown as tip sensor photo symbol), at the top 1 cm (yellow), the bottom 1 cm (brown), and midway between (orange). Drainage holes were located at the bottom of the column, where soil water can be sampled using a lysimeter indicated with photo symbol of suction 10 Rhizon SMS lysimeter (Rhizosphere Research Products B.V., Wageningen, The Netherlands).

4.1.5 Aims of this chapter: Measuring soil NO_3^- changes with sensors in forage crops in response to fertiliser application, defoliation, and intercropping

This chapter describes measuring soil N changes using NO_3^--selective sensors in response to cultivation of *L. perenne* in soil columns. Soil NO_3^- profiles at three depths were assessed under different management practices. These included varying NO_3^- application, defoliation of plant, and intercropping with the forage legume *M. sativa*. The sensors were tested for their agreement with conventional soil water analysis of NO_3^- in column drainage samples. This method was tested in columns to simulate the environment in the field, but with easier replication and standardisation of measurements.

The aim was to assess NO_3^--selective sensor suitability in forage crop studies of soil N profile changes using industry management practices. This will provide data for growers of forage crops on how their management practices may influence soil N levels for cultivation and any problems associated with fertiliser leaching.

4.1.6 Materials and Methods

The methods used in this chapter are detailed in Chapter 2, in section '2.2 NITRATE AVAILABILITY MONITORING USING SOIL SENSORS'. Supplemental data is provided in Appendix B.

4.2 RESULTS

4.2.1 Nitrate soil profiles of *Lolium perenne* columns vary with management practices

As a well-studied and important UK grass, *L. perenne* cv. AberMagic was tested in column experiments. The set-up for four columns was shown in Figure 4.3 with NO_3^--selective sensors in each depth, and the management practice for each column is indicated below in Table 4.1. One column had no *L. perenne* present, 'No crop', and three had *L. perenne* growing. These had either no NO_3^- application, 'Monocrop 1', had a NO_3^- application at day 0, 'Monocrop 2', or had NO_3^- application at day 0 plus aboveground tissue defoliation (crop harvest) and biomass measurement at day 28, 'Monocrop 3'. The industry regularly cuts their grass crop every 4 – 8 weeks, so 'Monocrop 3' represented two cuts in a cycle with 'Monocrop 1' and 'Monocrop 2' representing one cut. Seeding density matched industry standards, as did N applications. A settling period was included in experiments in order to check for problems with sensor failure before recording measurements. On day 0 columns were treated accordingly, and the crop grown over the next two months without interference apart from regular watering to near column capacity or saturation level. At the end of the two months in 'Monocrop' columns, the crop's whole vegetative tissue was cut, and the biomass measured, for 'Monocrop 3' day 28 biomass was included in the final total.

During the experiment data was captured periodically, and at the end only those sensors able to recalibrate to the defined standard (see Section 2.2.2) were used in analysis. The analysed data for four replicate columns of this experimental design are shown in Figure 4.4. Four graphs are shown, each corresponding to the column set-up indicated in Table 4.1, with each column profile depth level included. The first 'No crop' graph clearly shows the NO_3^- application at day 0 - 6 with an increase in sensor mM NO_3^- detected at the top of the column (yellow plot). From days 2 – 4 the NO_3^- application began to be detected in the middle of the column with a peak in mM detected at days 20 – 22 (orange plot), indicating leaching

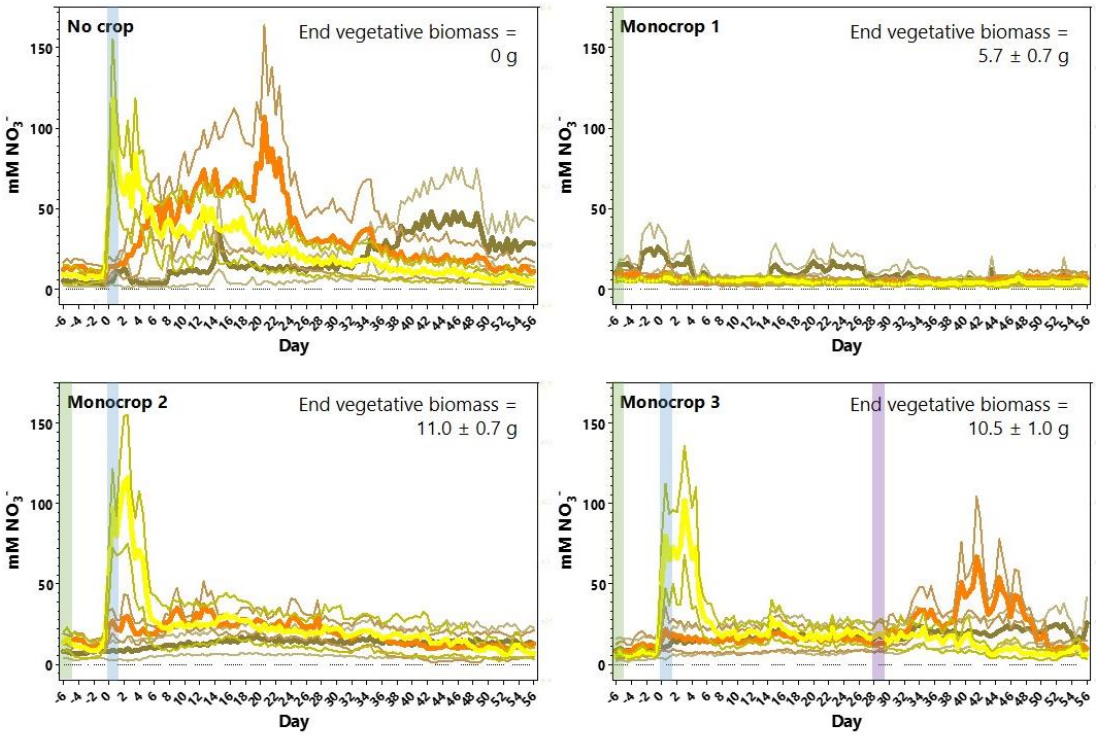
through the soil profile. From day 30 onwards the NO_3^- application was detected in the bottom level where mM increases were detected (brown plot). Diurnal changes in NO_3^- mM detected can be seen in this graph and all subsequent plots. In the next graph, 'Monocrop 1', no NO_3^- application was detected in any level, matching the management practice of no KNO_3 application of the column. The measured end vegetative biomass for this column was low at only 5.7 ± 0.7 g.

The third and fourth graphs show data for 'Monocrop 2' and 'Monocrop 3'. Again, in each of these the NO_3^- application was detected in the top level at days 0 – 6, (yellow plot), but quickly depleted with little increase in NO_3^- mM detected in the middle level from day 6 onwards. In both graphs the NO_3^- application was taken up by the crop, as no detected increase in mM was measured by the middle or bottom sensors. The crop uptake was also shown by the much higher end vegetative biomass measurements compared with 'Monocrop 1', with 10 – 11 g measured for each column. There was one major difference between 'Monocrop 2' and 'Monocrop 3' graphs, when defoliation had taken place there was seemingly a 'burst' of detected NO_3^- mM in the middle region only, (orange plot). To assess this burst in more detail further graphs were produced to show the data from each column level separately.

<u>Table 4.1:</u> Column set-up for the *Lolium perenne* monocropping experiment described in Section 2.2.4.

Columns were managed as colour coded and as follows; green for planting with 100 % *L. perenne* seedlings, cv. AberMagic, at seeding rate of 43.7 kg ha^{-1}; blue for day 0 nitrate application with KNO_3 treatments equivalent to 57 kg ha^{-1} as the standard in UK forage agriculture [157]; purple for day 28 total aboveground vegetative defoliation with biomass measurement. All columns with crops growing were cut on day 56 for biomass totals, and in the case of 'Monocrop 3' this was added to the day 28 measurement.

Column	Forage crop	N application	Cutting of crop
No crop		KNO_3 application	
Monocrop 1	*Lolium perenne*		
Monocrop 2	*Lolium perenne*	KNO_3 application	
Monocrop 3	*Lolium perenne*	KNO_3 application	Defoliation



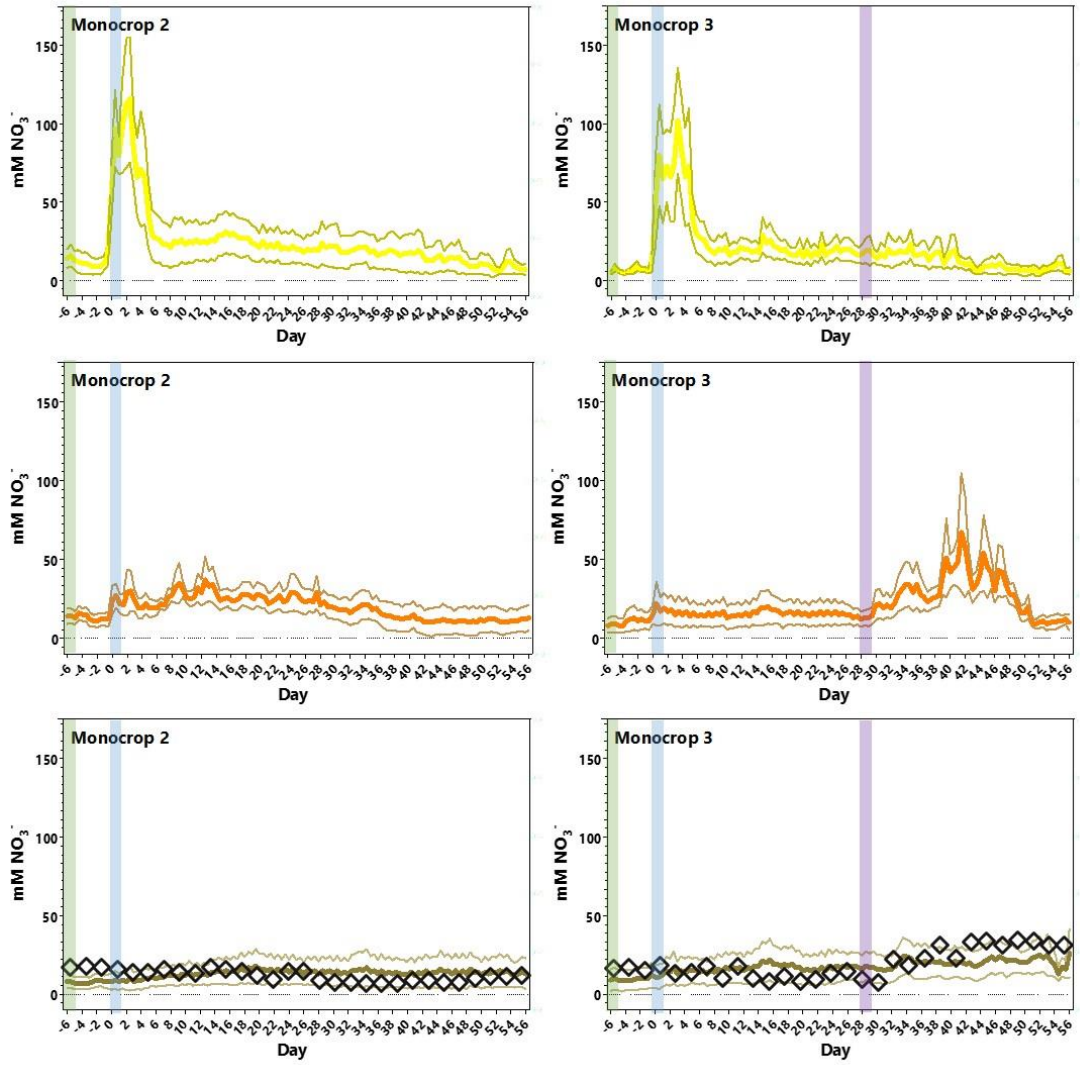
<u>Figure 4.4:</u> *Lolium perenne* monocrop column experiment NO_3^--selective sensor data.

Data are shown for each column separately, indicated in Table 4.1. NO_3^--selective sensor data are shown for top (yellow), middle (orange), and bottom (brown) levels of the columns, as described in Figure 4.3. Data is the 12 hour average of four experimental replicates in GraphPad Prism 7 (GraphPad Software Inc.), with standard error of the mean indicated with thinner lines of similar colour. Coloured vertical bars indicate the treatment for the *L. perenne* crop planted (green), nitrate application at day 0 (blue), or defoliation of total aboveground vegetative biomass at day 28 (purple). End total mean vegetative biomass is shown in g for each column with standard deviation of measurements across four experiments indicated.

4.2.1 Soil sensors detect a nitrate 'burst' in the mid column range following defoliation of *Lolium perenne*

Upon defoliation of *L. perenne* in a monocrop, sensors detected a delayed NO_3^- 'burst' in the middle section of roots, see Figure 4.4 'Monocrop 3'. Therefore, plots were produced to show each column level separately with further analysis. Figure B3 in Appendix B shows graphs for 'No crop' and 'Monocrop 1', indicating differences in detected NO_3^- mM from the graphs in Figure 4.4. Conventional soil water analysis of drainage hole leachate, indicated with black diamonds, showed good agreement with bottom sensor detected NO_3^- mM.

Figure 4.5 shows all levels separately for 'Monocrop 2' and 'Monocrop 3'. Figure 4.6 shows a repeated measurements ANOVA for significant differences between columns at each depth, which showed showed very low significant differences between detected NO_3^- mM in either top (a) or bottom c) levels for 12 h timepoints (red plots). However, the middle level 'burst' of increased NO_3^- noticed in Figure 4.4 was shown to be statistically significant in Figure 4.6 b) following defoliation, (blue plot), with $p < 0.05$ between days 39 – 48. The 'burst' disappears from day 48 onwards, with no significant change found in the bottom level subsequently (brown plot). Conventional soil water analysis of drainage hole leachate, (indicated with black diamonds), again showed good agreement with bottom sensor detected NO_3^- mM, although in 'Monocrop 3' this was slightly higher than the ion-selective sensor data.



<u>Figure 4.5:</u> Monocrop column experiment NO_3^--selective sensor data for 'Monocrop 2' and 'Monocrop 3'.

Column set-ups are described in Table 4.1. NO_3^--selective sensor data are shown independently for top (yellow), middle (orange), and bottom (brown) levels of the columns. Data are the 12 hour average of four experimental replicates in GraphPad Prism 7 (GraphPad Software Inc.), with standard error of the mean indicated with thinner lines of similar colour. Coloured vertical bars indicate management practice for the *L. perenne* crop planted (green), NO_3^- application at day 0 (blue), or defoliation of total aboveground vegetative biomass at day 28 (purple). In bottom level graph the soil water from drainage holes for one experiment was tested as leachate through conventional methods described in 2.1.15, indicated by black diamond symbols.

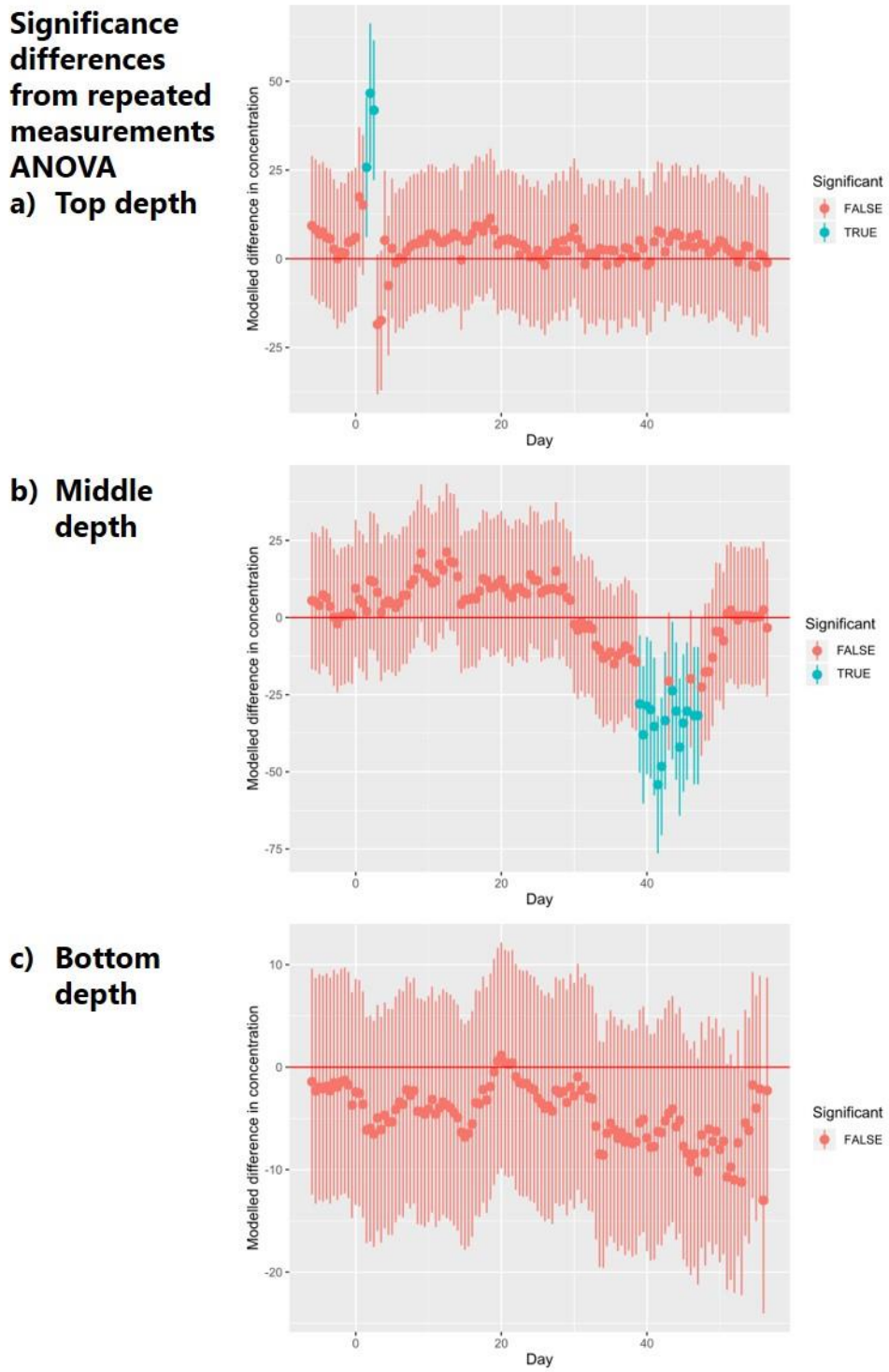


Figure 4.6: Repeated measurements ANOVA for NO_3^--selective sensor data for 'Monocrop 2' and 'Monocrop 3'.

Repeated measurements ANOVA conducted for differences between 'Monocrop 2' and 'Monocrop 3' in RStudio including time as a longitudinal factor. 12-hour timepoints not statistically significant are in red plots as 'FALSE', and those statistically significant with $p < 0.05$ are in blue plots as 'TRUE'.

4.2.3 Nitrate soil profiles were assessed when *Lolium perenne* columns were intercropped with *Medicago sativa*

Due to a push in modern agriculture to practice more sustainable intercropping, forage grasses and legumes (*L. perenne* cv. AberMagic and *M. sativa* cv. Daisy) were tested together in column experiments. The set-up of four columns is shown in Figure 4.3 with NO_3^--selective sensors in each depth, and the management practices for each column are indicated below in Table 4.2. One column had no crop present, labelled 'No crop', and three had *L. perenne* and *M. sativa* growing in an 80:20 mixed cultivation. These columns had either no NO_3^- application, 'Intercrop 1', with defoliation at day 28, 'Intercrop 2', or had a NO_3^- application at day 0 as well as all aboveground tissue defoliation, cut and measured for biomass on day 28, 'Intercrop 3'. Settling period, data analysis, and total end vegetative tissue measurements were carried out as in the monocropping experiments.

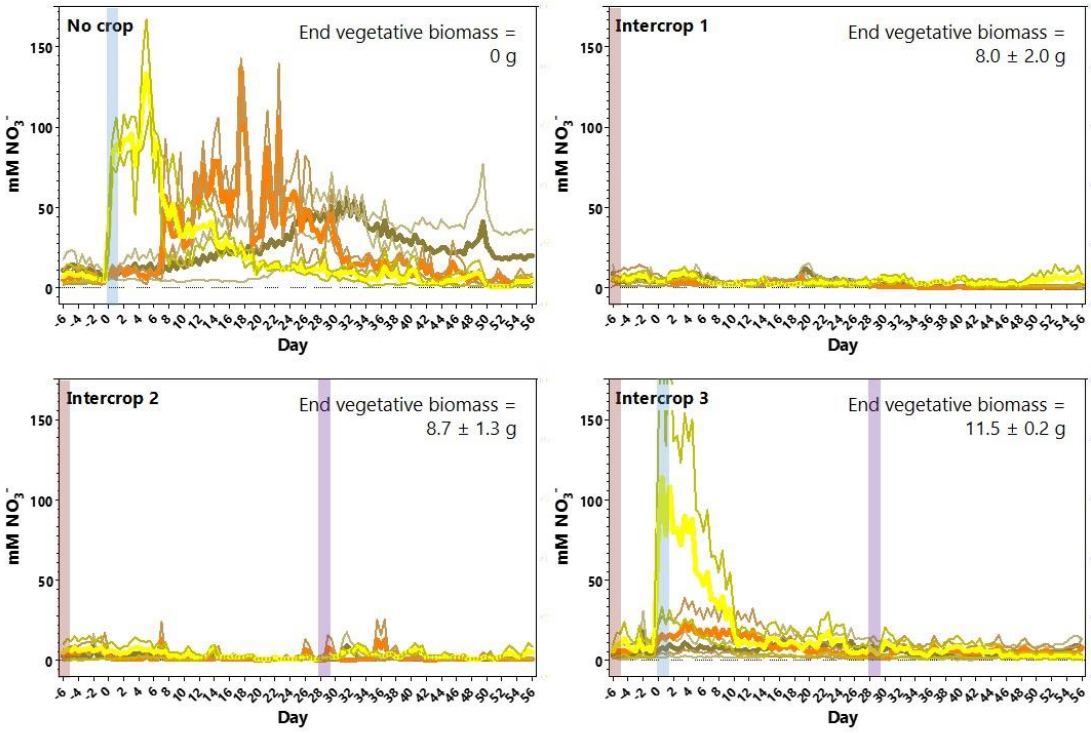
The analysed data for two experimental replicates of this experimental design are found in Figure 4.7. Two replicates were achieved in the available timeframe of the project, which may influence the standard error rate between averages. Again, four graphs are shown, each corresponding to the column set-up indicated in Table 4.2, with each column depth level included. The first 'No crop' graph showed the NO_3^- application at day 0 - 6 detected NO_3^- mM at the top of the column (yellow plot), with a later peak in the middle of the column shown at day 18 (orange plot). Slightly earlier from day 26 onwards the NO_3^- application was detected in the bottom level. This again indicated the NO_3^- application had leached through the soil profile. In the next graph, 'Intercrop 1', no NO_3^- application was detected in any level, this result is like that seen with 'Monocrop 1'. The measured harvest vegetative biomass for 'Intercrop 1' column was higher than that of 'Monocrop 1' at 8.0 ± 2.0 g. The graph for 'Intercrop 2' was similar to 'Intercrop 1' with no large change in the NO_3^- mM measured at any level, either before or after defoliation. The biomass for 'Intercrop 2' was similar to 'Intercrop 1', with a measurement of 8.7 ± 1.3 g.

The last graph for 'Intercrop 3' was similar to both 'Monocrop 2' and 'Monocrop 3', due to their similar management practices. The NO_3^- application was detected in the top level at days 0 – 6 (yellow plot) and quickly depleted with little increase in NO_3^- mM detected in middle level from day 6 onwards (orange plot). The harvest vegetative biomass measurement for 'Intercrop 3' was also like 'Monocrop 2' and 'Monocrop 3' shown in Figure 4.4. Despite having both an NO_3^- application and defoliation as in 'Monocrop 3', no NO_3^- 'burst' was detected in the middle region. Graphs were again drawn for more detailed analysis of the individual column depths separately.

Table 4.2: Column set-up for the *Lolium perenne* and *Medicago sativa* cv. Daisy intercropping experiment described in Section 2.2.5.

Columns underwent the management practice as colour coded as follows; pink for planting with 80:20 *L. perenne* cv. AberMagic and *M. sativa* cv. Daisy seedlings, at a seeding rate of 43.7 kg ha^{-1} to match the forage industry standard; blue for day 0 nitrate application with KNO_3 treatments equivalent to 57 kg ha^{-1} as the standard in UK forage agriculture [157]; purple for day 28 total aboveground vegetative defoliation with biomass measurement. All columns with crops growing were cut on day 56 for harvesting the biomass total, and in the case of 'Monocrop 3' this new growth mass was added to day 28 biomass measurement.

Column	Forage crop	N application	Cutting of crop
No crop		KNO_3 application	
Intercrop 1	*Lolium perenne* and *Medicago sativa*		
Intercrop 2	*Lolium perenne* and *Medicago sativa*		Defoliation
Intercrop 3	*Lolium perenne* and *Medicago sativa*	KNO_3 application	Defoliation



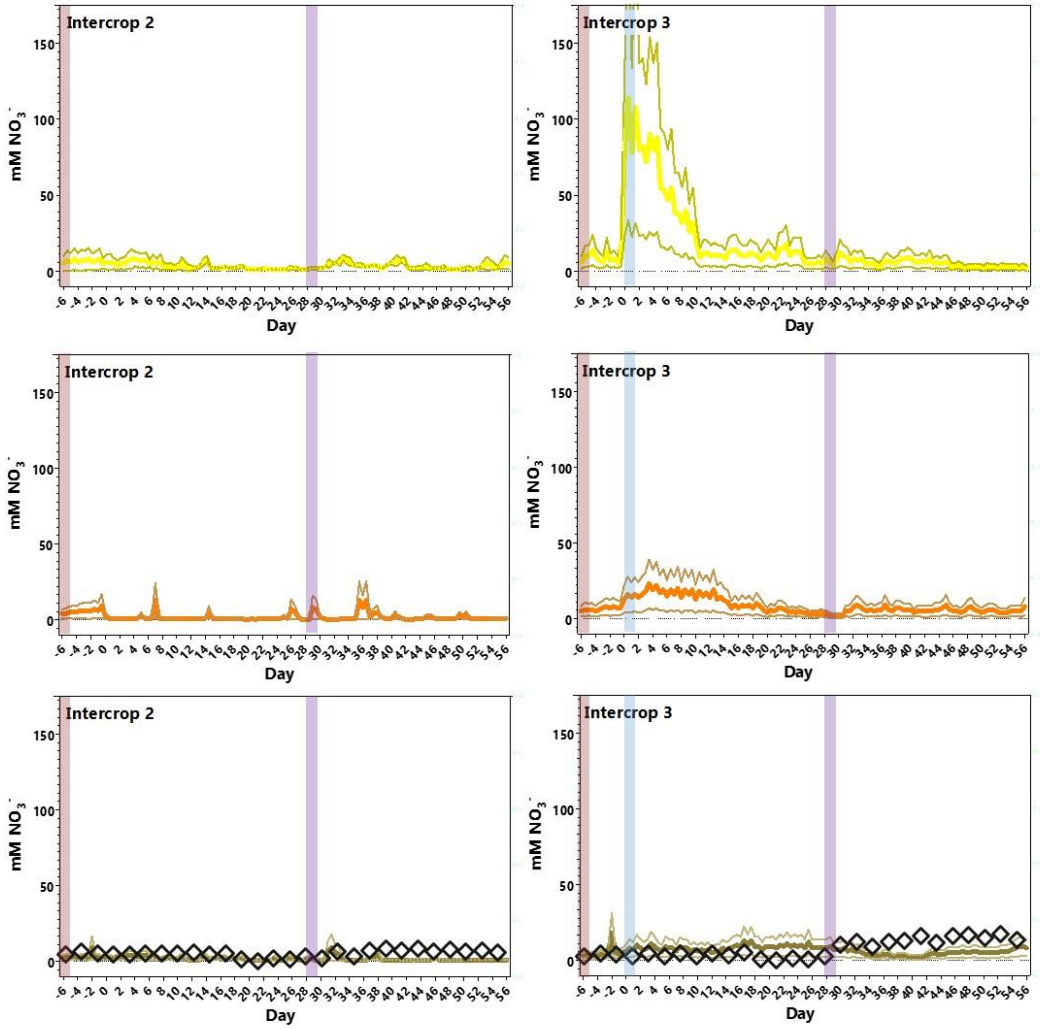
<u>Figure 4.7:</u> *Lolium perenne* and *Medicago sativa* intercrop column experiment NO_3^--selective sensor data.

Data are shown for each column separately, as indicated in Table 4.2. NO_3^--selective sensor data are shown for top (yellow), middle (orange), and bottom (brown) depths in the columns, as described in Figure 4.3. Data are the 12-hourly average of two experimental replicates in GraphPad Prism 7 (GraphPad Software Inc.), with standard error of the mean indicated with thinner lines of a similar colour. Coloured vertical bars indicate the management practices for *L. perenne* and *M. sativa* crops (pink), nitrate application at day 0 (blue), or defoliation of total aboveground vegetative biomass at day 28 (purple). Harvest total vegetative biomass is indicated in g for each column with standard deviation of mean measurements across four experiments indicated.

4.2.4 No nitrate 'burst' was detected following defoliation of *Lolium perenne* when intercropped with *Medicago sativa*

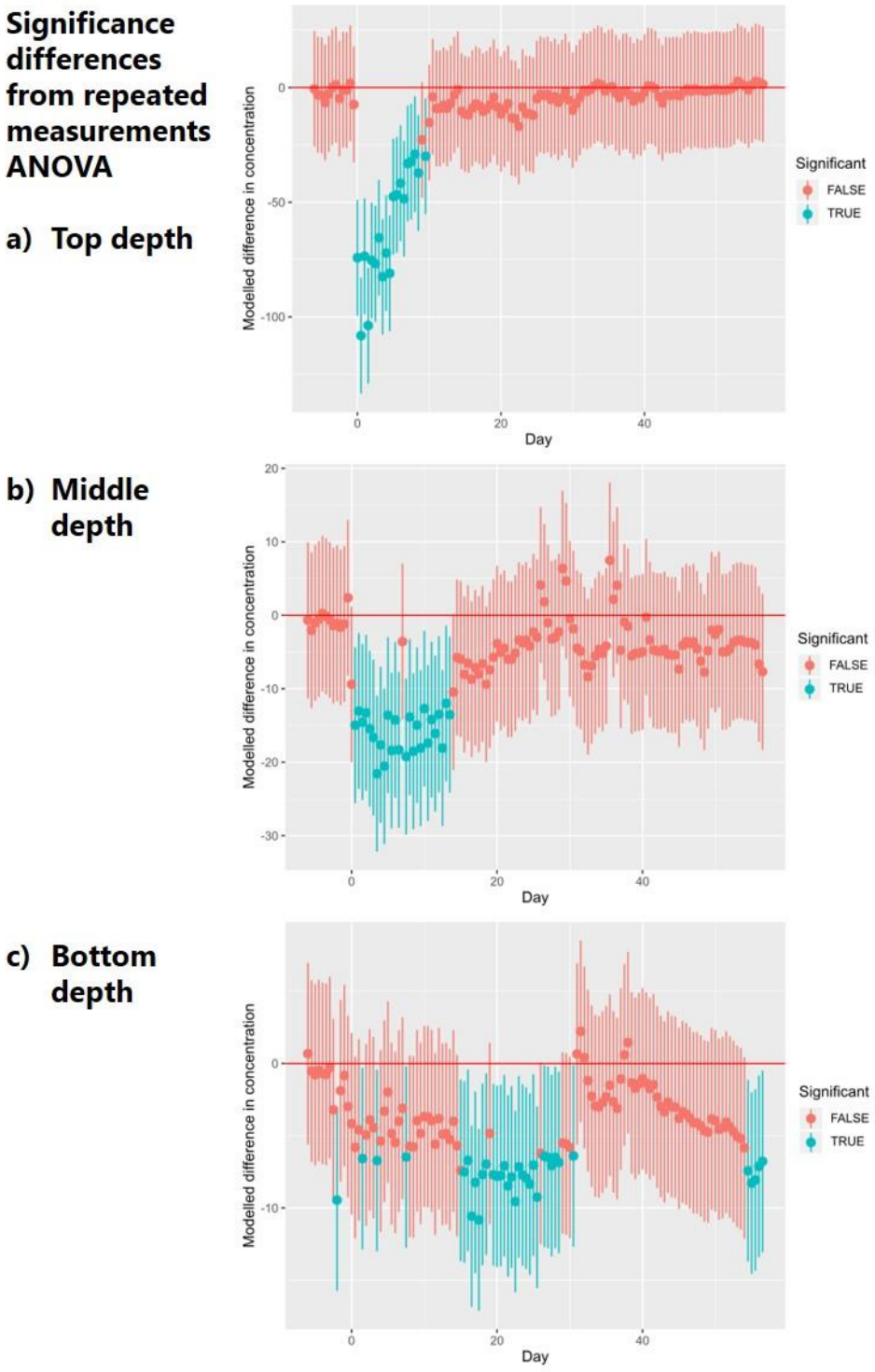
Upon defoliation of *L. perenne* in a monocrop, NO_3^--selective sensors detected a delayed NO_3^- 'burst' in the middle section of roots, shown in Figure 4.4 (orange plot). This was shown to be statistically significant when compared to non-defoliated crop, shown in Figure 4.6 b) (blue plots). This 'burst' was not detected at any depth under defoliated crops when *L. perenne* was grown alongside *M. sativa*, see Figure 4.7 'Intercrop 3'. Individual depth level graphs were again produced to check the significance of the findings described in Section 4.2.3. Figure B4 in Appendix B shows the graphs for 'No crop' and 'Intercrop 1'. In addition, conventional soil water analysis of drainage hole leachate (indicated with black diamonds) showed good agreement with the measurements reported by the bottom NO_3^--selective sensors.

Figure 4.8 shows all the depth levels plotted separately for 'Intercrop 2' and 'Intercrop 3'. Figure 4.9 shows a repeated measurements ANOVA for significant differences between columns at each depth, which showed significant differences between the detected NO_3^- mM in top a) level (blue plots) soon after the NO_3^- application from days 0 – 4. Other regions of significant difference are found in the middle b) level at days 10 – 13 where some leached NO_3^- application was detected by the sensors, and then again for a portion of the bottom c) level at days 15 – 31. There was little evidence of a NO_3^- 'burst' following defoliation, with or without a NO_3^- application. Again conventional soil water analysis of drainage hole leachate, (indicated with black diamonds on brown plot), showed good agreement with bottom sensor measurement of NO_3^- mM, although 'Intercrop 3' was slightly higher than NO_3^--selective sensor data.



<u>Figure 4.8:</u> Intercrop column experiment NO_3^--selective sensor data for 'Intercrop 2' and 'Intercrop 3'.

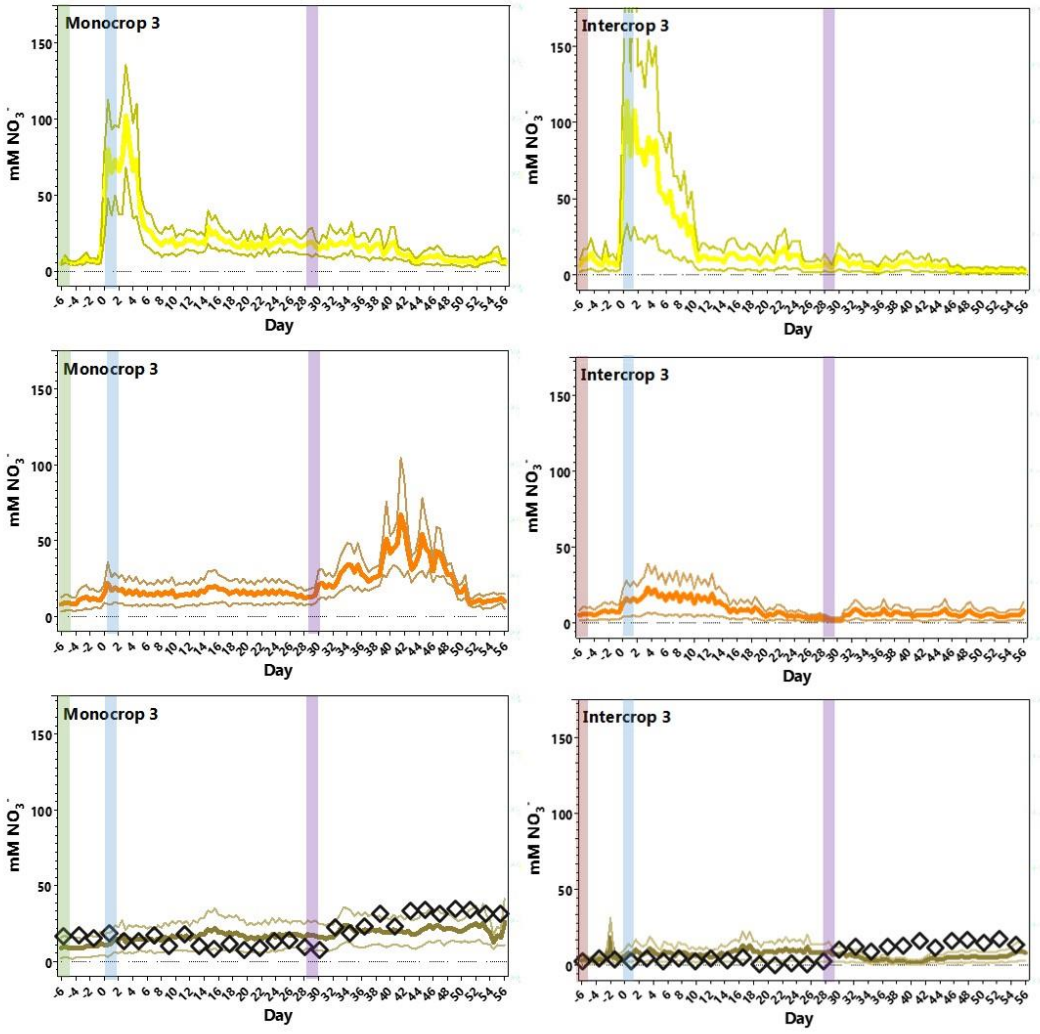
The column set-up was described in Table 4.2. NO_3^--selective sensor data are shown independently for top (yellow), middle (orange), and bottom (brown) levels of the columns. Data is the 12-hourly average of two experimental replicates, with standard error of the mean indicated with thinner lines of a similar colour. Coloured vertical bars indicate management practices for *L. perenne,* and *M. sativa* crop planted (pink), nitrate application at day 0 (blue), or defoliation of total aboveground vegetative biomass at day 28 (purple). In the bottom level graph, the soil water from drainage holes for one experiment was tested as leachate using conventional chemical assay methods described in Section 2.1.15, and indicated by black diamond symbol.



<u>Figure 4.9:</u> Repeated measurements ANOVAs for NO_3^--selective sensor data for 'Intercrop 2' and 'Intercrop 3'.

Repeated measurements ANOVA conducted for differences between 'Intercrop 2' and 'Intercrop 3' in RStudio including time as a longitudinal factor. 12-hour timepoints not statistically significant are in red plots as 'FALSE', and those statistically significant with $p < 0.05$ are in blue plots as 'TRUE'.

The differences between the cropping type, with all other management practices kept the same, was compared for 'Monocrop 3' with 'Intercrop 3'. This is shown for all column depth levels in Figure 4.10. These data show for top level (yellow plot) no significance apart from a small portion at the start of the experiment, likely due to the fewer replicates performed for 'Intercrop 3'. For the bottom level (brown plots) NO_3^--selective sensor detected mM was not different between plots, although 'Monocrop 3' look slightly higher in NO_3^- when observing conventional soil water analysis (black diamond symbols), but this cannot be tested accurately for significance with few data points. It was the middle level where there were differences in the plots, (orange plot). Again, no region of 'burst' in detected NO_3^- mM following defoliation can be seen in 'Intercrop 3' between days 34 – 44 when compared to 'Monocrop 3'.



<u>Figure 4.10:</u> Column experiment NO_3^--selective sensor data for 'Monocrop 3' and 'Intercrop 3'.

NO_3^--selective sensor data are shown separately for top (yellow), middle (orange), and bottom (brown) levels of the columns. These data are for columns of monocropping and intercropping experiments where KNO_3 application and defoliation occurred. Data are the 12-hourly average of four or two experimental replicates, with standard error of the mean indicated with thinner lines of similar colour. Coloured vertical bars indicate management practices for *L. perenne* only (green), or with *M. sativa* (pink), nitrate application at day 0 (blue), or defoliation of total aboveground vegetative biomass at day 28 (purple). In the bottom level graph, the soil water from drainage holes for one experiment was tested as leachate using the conventional chemical assay method described in Section 2.1.15 and indicated by black diamond symbol.

4.3 DISCUSSION

4.3.1 Soil columns can be used to produce standardised measurements of nitrate profiles

Column experiments allow the users to carry out multiple tests with defined parameters. This is similar to gel-based media systems and hydroponics, but soil columns allow for more environmental fluctuations, including N fertiliser leaching through the profile. Gel-based media systems are sterile and are in an environment with controlled light, temperature and humidity. Hydroponics are not sterile, but it is standard to change hydroponic solutions frequently to avoid unwanted pathogen effects and to provide nutritional repletion for adequate growth. An advantage of the soil column system is that the microbial populations, soil moisture levels, and light and temperature changes are more like those for crops growing in the field. These fluctuations may be less severe than field trials, but columns allow measurements to be standardised in laboratory settings to eliminate some of the climate variability occurring in field trials. This standardisation is important for testing the effect of different management schemes on NO_3^- leaching. This is especially true for forage grassland as there is a lack of evidence to suggest how different practices affect the environment [118], including the high intensity, temporary grassland like those schemes used by dried forage growers such as BAGCD.

As management practices can be changed relatively easily in columns, this work has shown that standardised measurements can be achieved in a relatively short time period compared to field trials. All NO_3^--selective sensor data for column types in this project show very similar measurements across replicate experiments, with minor standard error of the mean differences. It is true that NO_3^--selective sensors only measure discrete volumes of soil by testing the activity near to the sensors, however as there are multiple sensors at each depth, the data suggests these areas are representative of the whole depth volume. Moreover, the good agreement between experimental replicates, is shown in statistical testing between the measured NO_3^- mM, especially in Figures B3 and B4 of

Appendix B, suggesting the small volume tested by sensors over time is accurate for whole depth measurements. By having a soil based system with testing at numerous layers it is possible to see leaching in real-time after NO_3^- application in 'No crop' columns. The movement of NO_3^- through the soil profile is altered by the uptake of crop roots and by measuring the NO_3^- run-off from the base of the column it is possible to obtain a direct measure of the efficiency of the root system in acquiring NO_3^-. On this basis, the data suggest the forage crops grown in this work are efficient at obtaining NO_3^-.

4.3.2 Soil sensors match conventional measurements, but with higher definition and lower interference

Soil sensors can provide a wealth of data for cost-effective analysis of fields in real-time [517]. With these experiments, data from NO_3^--selective sensors built in the laboratory have been shown to provide similar measurements to conventional testing when analysing drainage hole leached soil water. Sensors have provided dramatically more detail with significantly lower labour when compared with conventional suction lysimeters or drainage water sampling. The NO_3^--selective sensor data can provide a more detailed picture of NO_3^- uptake by crops and soil microbes. This is particularly true for capturing diurnal changes in NO_3^-, for example in Figure 4.4 'No crop' plot. Such high definition of data throughout the day over a two and a half month period is unfeasible with current field methods. It is this wealth of data capture alongside low costs and labour which shows their superiority compared to the current testing methods described above. It is hoped that such testing in the future could aid quicker responses by the grower to improve growth conditions and combat leaching and future yield losses; this is very important for the success of precision agriculture [514; 517].

It should be noted that although feasible for short-term field use, see Figure 4.2, there are technical and durability problems with the NO_3^--selective sensors in their current design form for monitoring throughout an entire season. The NO_3^--selective membranes are very thin, about 1 –

2 mm thick, and so can easily be damaged especially in sandy soils. This damage can lead to the liquid backfill solution leaking, with evaporation from the top of the sensor providing another potential source of error due to inadequate sealing. Additionally, burying sensors in the field to depths > 50 cm can cause cracking of the plastic tips. The silver wire used for conductivity can corrode if sourced cheaply and the data loggers used can fail due to insufficient power supply. Finally, as the NO_3^--selective sensors are measuring soluble NO_3^- concentrations they are dependent on the soil water content, and extreme water conditions such as drought or flooding will change the NO_3^- concentration measured. For this reason, it is important to always include soil moisture measurements to monitor rainfall events. These factors mean that well-sourced and constructed sensors with rigorous lab testing work best in clay soils with adequate water drainage systems. Future sensors are likely to be made using solid-state technology like some new types of commercial pH sensors. These can offer the opportunity to avoid damage by soil particles and they may be used at greater depths.

4.3.3 The detected nitrate 'burst' following defoliation of *Lolium perenne*, but not when cultivated with *Medicago sativa*

The detected NO_3^- mM 'burst' found in the middle section of roots with NO_3^--selective sensors following defoliation of *L. perenne*, see Figures 4.4, 4.5 and 4.6, is very intriguing. A lack of NO_3^- uptake may have been expected [119], but a potential NO_3^- release is worthy of further investigation. There are hydroponics data to suggest N is released by roots at ~ 5.1 – 6.1% of the total plant storage under normal conditions [525], but this was not limited to one portion of the root under stress. It could be possible that this phenomenon is an experimental artefact, however this is unlikely as the experimental replicates show standardised measurements and no burst was observed when the grass and legume were intercropped. Possibly, the grass released NO_3^- as a stress response after cutting, or as a by-product of another similar process.

Post-defoliation, it is known that for the remnant aboveground vegetative tissue the uptake of carbon is stronger than for N [526], and therefore a release from the roots of NO_3^- may be due to this change in C:N ratio of tissue [527; 250]. Increasing defoliation of *Lolium* in hydroponics showed decreased plant N uptake and increased plant N remobilization [209], as shoots appear to be the predominant site of whole plant NO_3^- reduction in grasses [528]. Moreover, it is known that remobilization of plant C-containing compounds in the leaf is coordinated with N availability to the root [529]. However, hydroponic systems are unable to assess specific changes to root portions and importantly the soil microbial contribution is missing. As the detected mM NO_3^- 'burst' disappeared after day 48, shown in Figure 4.6 in 'Monocrop 3', any available N may be taken up by the plant once vegetative regrowth is established following defoliation, which is also shown in its relative end vegetative biomass total. Such changes may not be found in hydroponic analysis due to free movement of soluble NO_3^- through solution. Therefore, it may be possible for N-containing compounds to also be released from the plant as a stress response. Changes in both amide and amino acid composition has been found in *Lolium* xylem sap following defoliation [530], especially asparagine and glutamine, suggesting increased nitrogen assimilation [212; 531]. Moreover N reserves stored as vegetative storage proteins (VSPs) in roots and stem bases are rapidly degraded after defoliation [532]. But there is no published assessment to show if these N-compounds occur in root exudates.

It is documented that grasses in hydroponics can release carbon exudates from *Lolium* roots as a response to cutting [41; 42; 46], and also its close relative *Festuca* [43; 44; 47]. Alcohols and aldehydes can be released with complex profiles which change throughout developmental stage or cutting event for plant [533]. It could be possible that such C-containing compound exudates may cause a process to release NO_3^- in this middle region of the soil column. This process may be mediated by the plant or through microbial activity such as denitrifying bacteria. Carbon compounds and other non-nutrient root exudates are important as host specific recognition signals [534] and this may be the cause for microbial changes and their activity following defoliation. In permanent grassland it was

shown that the composition of microbial populations changed in response to defoliation [535], and although there was no effect on microbial activity, such population changes may affect the soil NO_3^- mM pools for this short 'burst' period. Moreover, possibly NO_3^- of microbial origin is not able to be taken up by the roots during this time due to changes in plant N uptake described above, so microbial processes are unaffected, and it is a plant only phenomena causing this 'burst'.

As the 'burst' is not found when intercropping with *M. sativa* is performed, then a microbial change caused by legume root presence may be possible; it is known that there is greater diversity in soil microbial populations for legumes when compared with grasses [536; 537]. However, this may be due to the differences in root architecture of legumes when compared to grasses that may be important for this NO_3^- 'burst'. Changes in transpiration rate post-defoliation are recorded in many plants [538-542] and these may affect certain portions of the root to different amounts, however more work on root differences is required for such hypotheses. Specific species and plant taxa vary in their root branching shapes [543] with plant root ideotypes having slightly different patterns that may be important for breeders [544]. The differences found in the long tap root grasses, including *Lolium* may be influence the N profile changes when compared to the much more branched legume *Medicago* [545; 546; 478], and thus could be investigated in more intercropping experiments for leaching investigations.

Chapter 5: Fulvic acid increases vegetative growth in the forage legume *Medicago sativa*, and is associated with influencing microbial activity

5.1 INTRODUCTION

5.1.1 Using biostimulants to aid crop yields

During the past 100 years of modern agriculture, crop production has become dominated by intense agricultural practices. These have included concentrated mono-cropping with increasing use of fertilisers, pesticides, and invasive soil treatments including high-tillage [547]. Such practices can have significant negative environmental impacts, including excessive accumulation of nutrients and contamination of drainage water [548; 549; 483; 550]. Intensive agricultural practices can also depreciate soil through erosion, C and N losses, and lowered microbial activity [88; 551; 405; 552; 553]. These practices also affect greenhouse gas emissions across the UK and Europe [554; 555; 452; 453; 556; 456; 557; 558]. Due to the problems with modern, intensive practices, studies with a focus on alternative methods are being investigated. Forage crops cultivated for dried feed have huge N fertiliser inputs, which is discussed in detail in Appendix E: 'UK dried forage production: a review of industry changes and assessment of prospects for both policy and science'. New management practices for forage crops that can reduce the negative impacts of cultivation are required.

Improvements in plant nutrient capture efficiencies are being investigated, with the aim of reducing fertiliser use without impacting on yield. Often this is achieved through the use of breeding [338; 328; 339; 559; 560; 14; 3; 335; 331; 336; 337], or genetic manipulation [561-563; 373; 564-572], to expand productivity. However, there are time limits in both breeding and transgenic techniques in forage crops, which mean application of research to growers is slower than other crops [573]. Studies with faster applicable results include investigating forage crop rotation changes, particularly of forage legumes, on farms [574-580] and changes to tillage management [111; 581-583; 89; 584]. These studies have emphasised a need for yield improvements, but also a push for more sustainable agriculture, focusing on not only the plant, but also the soil and the wider biodiversity reaction to management [103; 105; 585-591].

One strategy which may improve forage plant performance is the use of molecules that act as biostimulants. As defined by Du Jardin in 2015, biostimulants correspond to any substance applied to plants to enhance nutrient efficiency regardless of nutrient content, and thus do not include fertilisers and pesticides [120]. The investigation of potential biostimulants is a focus point in current plant science including in forage crop research, and can incorporate studying the use of biotic protein hydrolysates [592-594], plant growth-promoting rhizobacteria (PGPRs), and beneficial fungi [595-599], algae extracts [600; 601], and inorganic salts (e.g. Al, Co, Na, Se, Si) and amino acids [602; 120; 603; 604]. Such potential biostimulants include humic substances (HS), including specifically the derivative fulvic acid (FA).

5.1.2 Humic substances are potential biostimulants, and include fulvic acid

Humus accounts for ~ 10 % of soil content across terrestrial ecosystems and it is also found in rivers and wastewater, with significantly lower levels in agricultural land [605]. Humus is formed of decaying biological tissue, mainly of plant origin. Insoluble humin and extractable humic substances (HS) are a major component of humus. Extractable HS fractions are humic acid and fulvic acid (FA), and these are considered key soil components as their complex composition may be responsible for facilitating many complex chemical reactions in soil systems [606-609]. All fractions are a poorly characterised mixture of chemicals, and although many have been shown to increase growth parameters in numerous species [123; 610; 611; 122; 612; 613], their mode of action is unclear. There is some debate over which fractions, the high or the low molecular weight, are the most active. Canellas *et al*., (2010) concluded it is the lowest molecular masses, although indicated that this may be less to do with size, and more their specific bioactive molecule content [122]. The lowest molecular weights are FAs [614; 607], which unlike humic acid are water soluble across a wide range of pH, and so will be the focus of this study.

Despite the wide variety of sources of FA from temperate to tropical soils to Leonardite waste material, there is a uniformity in the gross properties of HS [615-617]. Analyses provide evidence that all FA are complex formulations of C-containing compounds, with potential metal-binding capacities [618; 619; 614; 620; 606; 621; 607; 609; 622]. Biochemical analyses over the decades has demonstrated that these products are supramolecular associations of many compounds whose characteristics depend on their level of aggregation [606]. These compounds are organic, but cannot be classified into other groups such as proteins or starches. The extraction procedure for FAs has varied over the last 200 years, but generally involves acidification to pH 2 followed by precipitation, with the solid precipitate purified by absorption through XAD-8 resin or dialysis [623-626]. The precipitant gained and the method of purification have a large influence on the characteristics of the resulting solution and the extraction methods may differ in their component steps [626-629]. Extraction methods are under ongoing review with patents being awarded that depend on the possible future applications of the humic derivatives. The possible applications for FAs are wide-ranging, from medicinal studies into wound healing [630], to studying complex metal chelation interactions such as for cadmium, lead and copper [614; 631; 632], and crucially for this study in improving crop quality and yield.

5.1.3 Studies have investigated humic and fulvic acids effects on plant growth

Many studies have looked into the benefits of applying HS, as humic acid or FA extractions, in either the growth medium or as a foliar application to a wide range of crops. In *Arabidopsis thaliana* and many cereal crops, HS has been shown to have effects on plant growth including increased root architecture, improved nutrient uptake and yield even under stress, and enhanced access to metals [633-640], with comprehensive examples detailed in numerous reviews [641-643; 610; 644; 613].

A study of particular prominence to forage crops is that of the legumes soybean (*Glycine max*), peanut (*Arachis hypogaea*) and clover (*Trifolium*

vesiculosum) [645]. This study showed that a sand growth medium supplemented with FA increased nodulation weight, but not size, in a dose-dependent manner. Application of HS in *Pisum sativum* also showed an increase in root nodulation and mycorrhizal colonisation [646]. If such increases were able to improve N fixation in legumes, then this could in-turn increase the N storage of their vegetative tissue. The important forage crop *Medicago sativa* (commonly called 'lucerne' and 'alfalfa') has been found to increase in vegetative biomass with FA application, however with variable responses in different conditions [647; 648]. Studies using various HS including FAs have been carried out in other important legumes and forage grasses [124; 649-652], however, these experiments did not include appropriate nutritional controls and more detailed studies are required to assess the effect of FA on forage crops.

5.1.4 Limited evidence for the mode of action of humic substances

Identification of the specific effects of humic substances requires well structured, specific methods that are not always met in published research. Research on FA is often limited by the chemical analysis and frequently using samples which are not easily replicable, because their source is unique [633]. This makes designing appropriate controls for experiments difficult. Due to this lack of analysis many studies often rely on a 'no application' or 'water treatment' for controls, to determine the FAs potential biostimulant effect on a plant [610].

Some studies have linked HS and extractions including FA with hormone-like responses in plants, particularly *A. thaliana* [653; 654; 643; 655; 121], however there is a void of evidence from well-controlled experiments to support this. Other than this tentative hormonal hypothesis as a mode of action, most studies conclude that it is the complex composition of HS that promotes the above changes in physiology, nutrient uptake, or stress response. This is understandable, due to the nature of elucidating a single growth response from substances so complex in their composition. It is

imperative that more studies of FA are carried out with good controls to elucidate the role it may play in improving crop growth.

5.1.5 Aims of this chapter: Assessing the potential biostimulant fulvic acid in forage crops

This chapter describes work investigating the use of FA to promote growth in the cultivation of forage crops. This will be useful for forage growers to assess if inclusion of this treatment can increase the yields of their crops, without increasing N fertiliser inputs. Two readily-available, commercial FAs will be tested across a range of important temperate forage crops. One of the major problems in analysis of FA effects on plant growth is that studies have not attempted to control for nutritional composition of FA treatment. Adequate chemical analysis of the FA used is often lacking and so will be incorporated into this project, including controlling for nutritional composition.

Treatments are first assessed in the glasshouse to establish growth effects on crops, before being trialled in the field. Field trials were at forage grower sites with applications and management using industry standard practices. The aim was to identify if a change in management practice, including FA treatments, could be used in UK forage cultivation under conventional farming methods.

5.1.6 Materials and Methods

The methods used in his chapter are detailed in Chapter 2, in section '2.3 FULVIC ACID TESTING OF FORAGE CROPS'. Supplemental data is provided in Appendix C.

5.2 RESULTS

5.2.1 A screen of two fulvic acid applications found a vegetative yield increase in forage legumes, but not in grasses

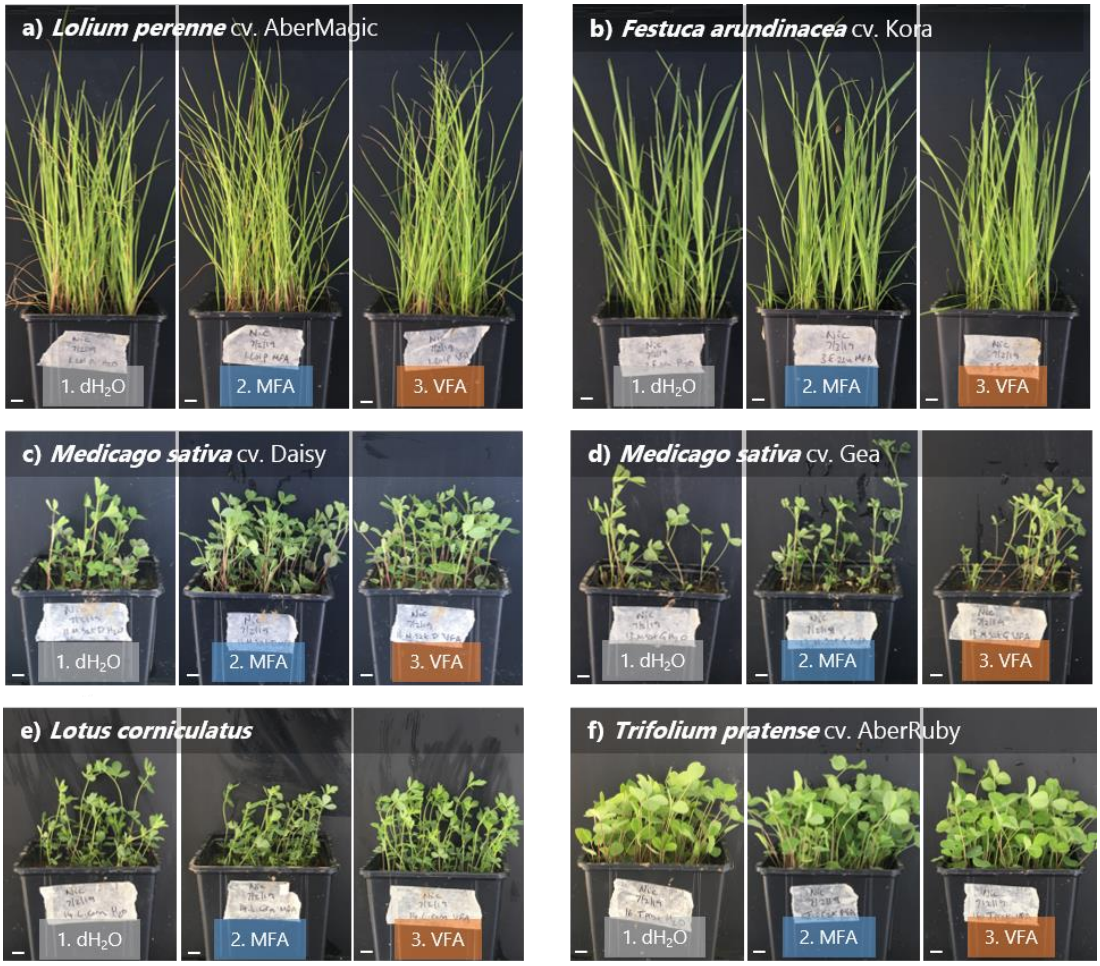
Two commercially available fulvic acids (MFA and VFA) sourced from the UK and USA were tested with forage crops to see if they improved their vegetative yield. Fifteen forage crop species and cultivars important in UK cultivation were tested in the glasshouse, with details found in Tables 2.4 and 2.5. Crops were treated either with the FA or with deionised water (dH_2O), and the vegetative yield was measured as the total aboveground biomass after 3 weeks of growth. Figure 5.1 shows yields for 6 cultivars, the grasses *Lolium perenne* cv. AberMagic and *Festuca arundinacea* cv. Kora (a and b), and the legumes *Medicago sativa* cv. Daisy and cv. Gea, *Lotus corniculatus*, and *Trifolium pratense* cv. AberRuby (c, d, e, and f). Grass yields did not visually differ between treatments, whereas MFA and VFA treated legumes all looked notably bushier with more vegetative production.

Biomass measurements for the FAs treatments were calculated relative to the dH_2O (as 100 %) and compared across experiments, as shown in Figure 2.5. There were no significant differences in biomass for grasses with application of FAs, matching the visual observations in Figure 5.1 a) and b). There is a slight statistically significant measurement for *Anthoxanthum aristatum* with MFA as p-value < 0.05, but this difference is small and may not be enough to suggest that FAs are capable of increasing grass yields. A two-way ANOVA showed that neither FA treatment was statistically significant in increasing biomass, indicated with significance letters.

On the other hand, Figure 5.2 shows that legume biomasses were dramatically increased by FA application, particularly *M. sativa* cv. Daisy and Luzelle, and *L. corniculatus*; two-tailed student t-tests indicated in graphs for most measurements of FA applications were $p < 0.05$, and if a

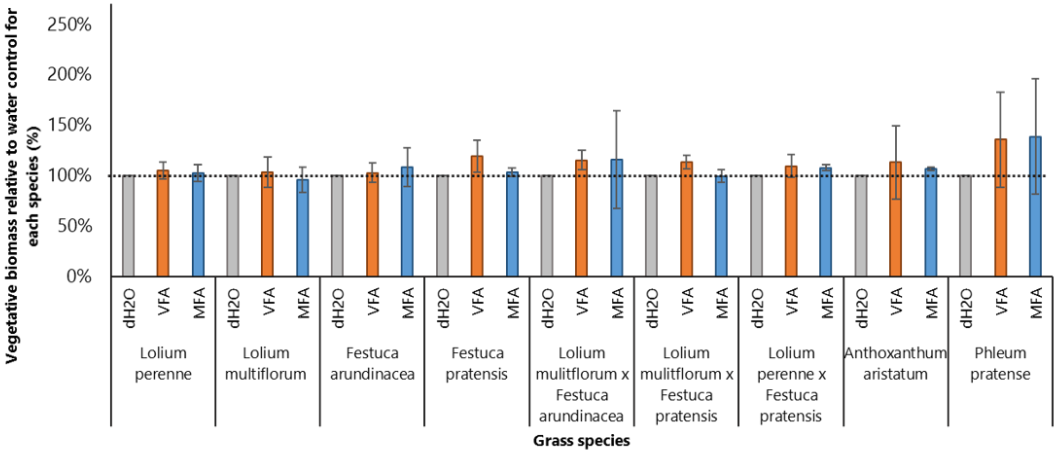
one-tailed test was used based on visual observations then all measurements are statistically significant for FA application. This was also demonstrated in visual observations of the leguminous crops shown in Figure 5.1 c) – f). A two-way ANOVA showed that both FA treatment were statistically significant in increasing biomass, indicated with significance letters. As *M. sativa* is a widely cultivated forage crop, the vegetative growth increase measured is commercially important and requiring further assessment.

As farm-collected soil was used in these experiments, the effect of FA treatment on weed count rate and final soil pH was compared, and is shown in Figure C1, Appendix C. FA treated pots had higher numbers of leafy, dicot weeds during the first week post treatment when compared to dH_2O control (b) and more grass weeds like couch grass at the start of the second week (a). This supports other documented cases in the literature of HS inducing germination across many plants species [656; 619; 657; 658; 650]. Final pH of soil was also higher in FA treated pots, especially those with MFA treatment. Similarly this matches other published evidence that humic substances can act as soil conditioners, reducing acidity through increasing soil aggregate stability and reducing ionic species [618; 614; 620; 621]. Chemical analysis of both types of FAs was required to determine how similar their compositions and identify any similarities.



<u>Figure 5.1:</u> Vegetative tissue of forage crops following one of two fulvic acid treatments compared to a control.

Treatments were applied to seedlings at 12 days post germination and vegetative yields were assessed at 21 days post treatment. Treatments were deionised water (1. dH_2O in grey), 0.5 % MFA (2. MFA in blue), or 1 % VFA (3. VFA in orange). Fifteen forage crop species/cultivars were tested in total, with the above showing two grasses and four legumes; the grasses were a)-b), *Lolium perenne* cv. AberMagic and *Festuca arundinacea* cv. Kora respectively; the legumes were c)-f), *Medicago sativa* cv. Daisy and cv. Gea, *Lotus corniculatus*, and *Trifolium pratense* cv. AberRuby. Scale bars indicated 1 cm.

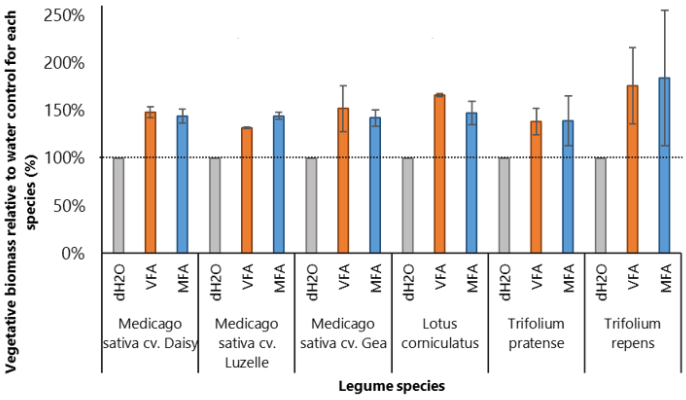


Two-way factor ANOVA – Grasses

	Treatment	Cultivar	Both
rep.	18	6	2
d.f.	27	27	27
s.e.d.	0.0665	0.1151	0.1994
	Mean	Significance level of 0.05	
dH_2O	1	a	
MFA	1.085	a	
VFA	1.127	a	

Two-way factor ANOVA – Legumes

	Treatment	Cultivar	Both
rep.	12	6	2
d.f.	18	18	18
s.e.d.	0.0879	0.1243	0.2154
	Mean	Significance level of 0.05	
dH_2O	1	a	
MFA	1.498	b	
VFA	1.517	b	



<u>Figure 5.2:</u> Vegetative biomass of forage crops following one of two fulvic acid treatments relative to a control.

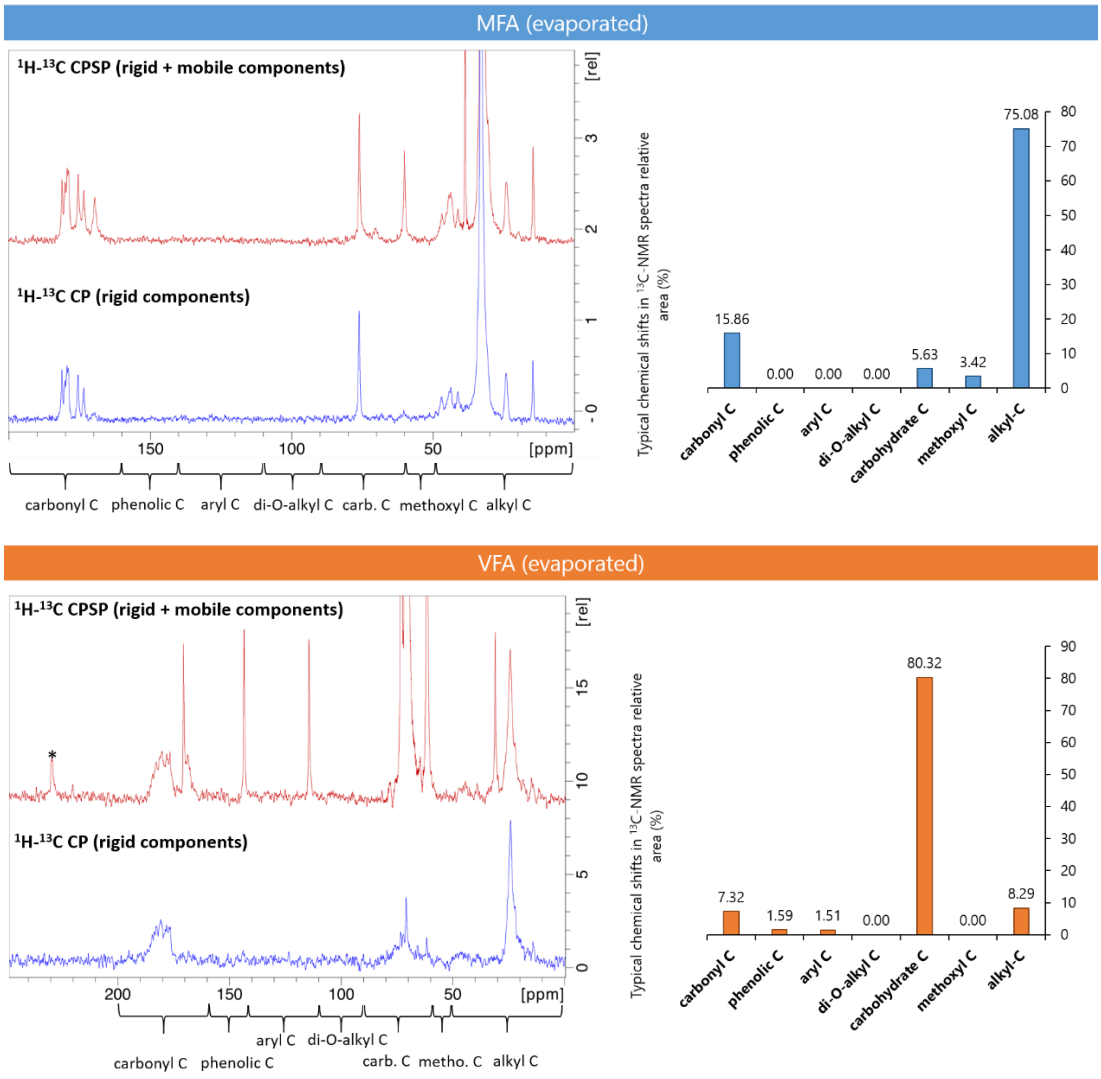
Treatments were applied to seedlings at 7 days post germination and vegetative yields were assessed at 21 days post treatment. Treatments were deionised water (dH_2O in grey), 0.5 % MFA (MFA in blue), or 1 % VFA (VFA in orange). Fifteen forage crop species/cultivars were tested in total, with the top bar chart showing grass species, and the below bar chart showing legumes. Biomass was measured for two independent experiments with biomass for both FAs calculated relative to dH_2O (shown as 100%). Error bars show standard deviation between experiments. A two-factor ANOVA was also performed for grass and legume cultivars, as displayed in table with significance level of 0.05.

5.2.2 Analysis of fulvic acids found varying chemical composition

Although FAs have shown both in this study and in other literature to affect numerous crops including legumes, it is not clear whether the effect is as a biostimulant, or as a nutritional additive. Analysis of FA composition was necessary to elucidate whether application is working actively to effect growth, or whether it is a fertiliser.

Using ion-coupled plasma (ICP) analysis, a range of metal ions were detected, as well as total N and C contents; these can be found in Tables 2.8 and 2.9. Several ions were found at very low concentrations and varied between FAs; MFA included detectable Fe, with trace amounts of Ti, Mo, Cr, Cd, P, Zn, Co, Ni, Cu, Mn; VFA included detectable Ca, K, Mg, S, and Na, with trace rates of Al, Co, Cu, Fe, Mn, Ni, Zn. FAs included total N of 1.51 and 8.59 mg/L respectively, with both FAs containing C compounds but VFA to a detectable limit of 0.03 mg/L. Although elements including metals were detected, they may not be in high enough concentrations to be a source for the plant; for example in MFA Fe content was 839 ppm, but a normal range in soils is 7000 – 55000 ppm dry weight [659; 660]. It was unclear whether some of these metals were chelated, but they make up a small but significant portion of both types of FAs treatment. These elemental measurements should be included in 'control' treatment solutions. These new nutritional controls were called MC for MFA and VC for VFA, and these were used in future experiments (see below).

NMR was implemented to compare the organic compounds found in MFA and VFA. Spectra of rigid and mobile C components using ^{13}C-NMR is shown in Figure 5.3 below, alongside abundances compared to total of each class of compounds. The most striking difference between FA samples was that MFA largely contained alkyl C compounds (75.08 %), whereas VFA contained largely carbohydrates as saccharides (80.32 %). Both shared a substantial portion of their organic matter as carbonyl C (15.86 and 7.32 %). This data shows FAs can have very different compositions of organic matter, which matches their variation in metal ion contents above.



<u>Figure 5.3:</u> Analysis of two fulvic acids (MFA and VFA) using ^{13}C-NMR for carbon compounds in evaporated samples.

Analysis was performed on evaporated MFA (blue) and VFA (orange). NMR spectra is shown for both on the left hand side, for rigid and mobile components (red traces), and for rigid components only (blue traces). Below traces there is a guide for which carbon compound type is found at that chemical shift range in ppm as follows; carbonyl C in 160 – 220 ppm (C=O including ketones, aldehydes, acids, and esters); phenolic C in 140 – 160 ppm and aryl C in 110 – 140 ppm (aromatic rings); di-O-alkyl C in 90 – 110 ppm and carbohydrate C in 60 – 90 ppm (primarily alkanes); methoxyl C in 50 – 60 ppm (compounds with methyl group bound to oxygen); alkyl C in 0 – 50 ppm (alkanes). The relative composition of carbon compounds is shown on the right hand side as bar graphs, with values indicated. '*' is an unknown peak in VFA rigid and mobile components trace and is not included in total relative composition. Experiment and data analysis performed by Dr Juan Carlos Muñoz-Garcia, School of Pharmacy, University of East Anglia).

Finally, using mass spectrometry (MS) techniques a small number of compounds were detected in FAs to include in control solutions. In MFA, citric acid derivatives were detected, see GC-MS spectra in Figure C2, Appendix C. Derivatives detected matched to citric acid in both chromatograms with citric monohydrate standard, and with library derivative entries (93.3 % and 19.8 %). These derivatives were isomers of citric acid including free R-(homo)2-citrate, likely to be found in a complex with Fe^{2+}, which is in high levels when compared to other elements. It was unsure whether citrate and iron would form a complex in MFA strong enough to remain associated throughout chromatography or whether the complex dissociated during chromatography, with the separated ions citrate, homocitrate, and (homo)2-citrate all detected. It does not functionally matter whether the complex only formed in the spray chamber, or not. What is relevant is whether citrate/homo-citrate from MFA is capable of forming a complex with transition metals in soil conditions, and whether this is beneficial to plants, for example by increasing availability of trace nutrients. It was therefore important that citrate was included in our control solution MC to test such capabilities. It is worth noting citrates are exuded by the roots of some plants for metal chelating effects [661], so it is entirely plausibly that MFA could also be showing this chemistry for metal acquisition.

In VFA, a low weight polyethylene glycol (PEG) cluster was detected, see GC-MS spectra in Figure C3, Appendix C. The cluster detected matched to PEG in both chromatograms with a PEG-400 standard, and with a library entry match (93.43 %). It was unsure whether PEG in VFA was either present in FA starting humic material, added during the extraction process, or if is a by-product of the extraction. PEG is known to induce water stress effects in plants, but only when molecular weights and concentrations present are high enough. Although PEG-400 can penetrate plant tissues when grown in solution, leaves and root systems will not be damaged unless grown in a concentrated PEG solution of at least 100 mL/L and with a low osmotic pressure (-14 bar) applied. Even so, this concentration of PEG is low with only small amounts of local damage occurring [662]. In VFA PEG concentration is only approximately 1.5 mL/L. It is unlikely to be causing a water stress effect, however as this was identified in VFA and

stress effects have been noted in the literature, PEG-400 was included in control solution VC.

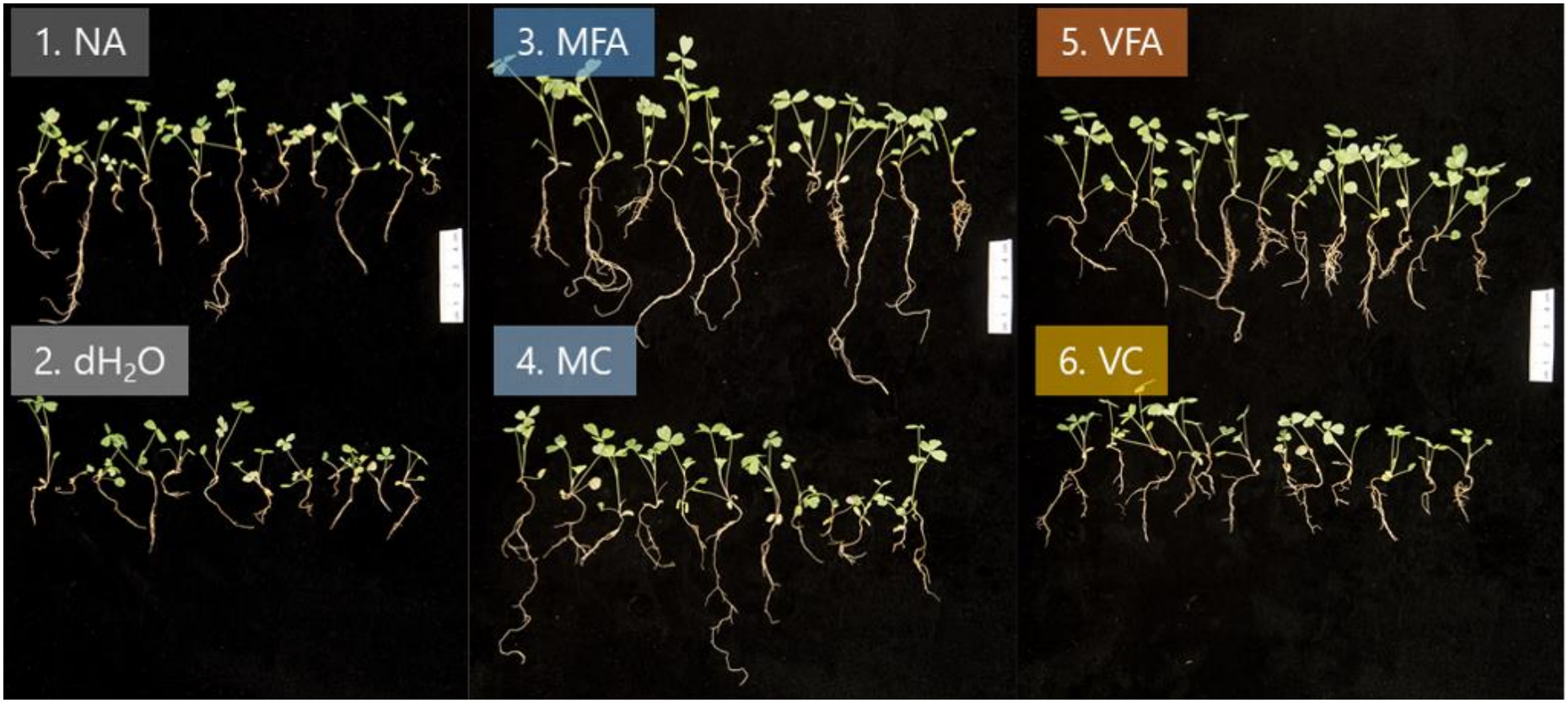
Also in VFA, derivatised isothiazolone was detected in very low abundance (PEG:isothiazolone abundance ratio was 20:1), with a database match of 97.2 %. Isothiazolone is a biocide and generally used for water treatment and may have been added to keep the product free from bacterial growth [663]. A benefit of isothiazolone over other biocides is that it stays stable in an acid environment [664] which is important when soil acidity is of increasing concern for growers. It was not deemed necessary to include isothiazolone in our control solution because it was found in such low abundance. Moreover, it was probably added to VFA keep the product free of microbial contamination, however in this study there was no requirement for this extra level of control, as all solutions were sterilised by autoclaving before the plant assays.

This analysis alongside ICP and MS shows how two FAs can have very different compositions, despite having similar starting material and following the same extraction process. Therefore, it is imperative that as much of the contents of FAs as feasible are added to controls in plant assays. This will determine if FAs are acting on plants through a specific pathway with an active ingredient, or just as an extra nutritional addition. The controls for MFA and VFA are termed MC and VC respectively, and will be used in subsequent assays.

5.2.3 Fulvic acid increase growth of *Medicago sativa* and is specific to its composition and not only a nutritional effect

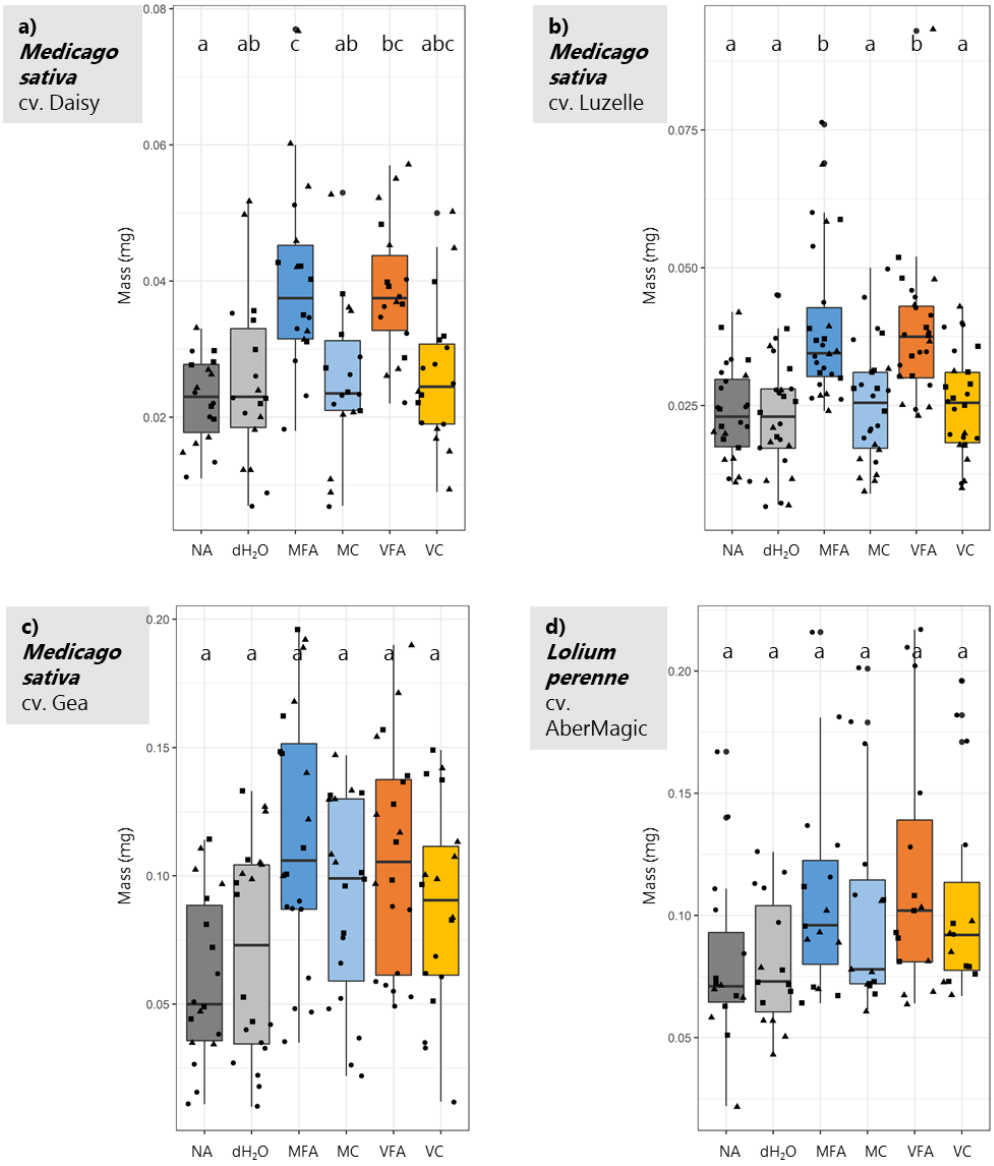
Further biomass yield assays were carried out in the glasshouse with *M. sativa* cultivars using applications of MFA and VFA alongside their control solutions MC and VC (see 5.2.2). Moreover, controls of no application (NA) and deionised water (dH_2O) were also included to ensure there was no water availability factor involved in the growth increases. Figure 5.4 shows visually FA-induced vegetative growth stimulation after three weeks in *M. sativa* cv. Daisy. Both MFA and VFA have increased vegetative biomass compared to controls, with plants taller and bushier with more lateral expansion of shoots and leaves. There was no difference in root biomass between treatments.

Vegetative biomass measurements were recorded in three independent experiments for cv. Daisy, Luzelle, and Gea. Results are shown in Figure 5.5, alongside the grass *L. perenne* cv. AberMagic. Both cv. Daisy and Luzelle showed significantly increased vegetative growth when compared to control solutions; cv. Gea showed more growth but this increase was not statistically significant (see ANOVA letters of Figure 5.5c, with significance level of 0.05). This is likely due to the fact cv. Gea was tested throughout the winter months in the glasshouse, when temperature fluctuated more rapidly, and there was more variation between experiments (as shown in individual sample points). If relative increase in growth in independent experiments is used to assess yield increase, then the biomass of all cultivars was significantly increased with FA application compared to all controls. When using both raw yield values as in Figure 5.5, or if using relative experimental increases, the grass *L. perenne* did not show a significant growth increase with FA treatment.



<u>Figure 5.4:</u> ***Medicago sativa*** **cv. Daisy seedlings following treatment with fulvic acids or controls.**

Treatments were applied to seedlings at 7 days post germination and photographs above were taken at 21 days post treatment. Treatments were; no addition (1. NA in dark grey); deionised water (2. dH_2O in grey); 0.5 % MFA (3. MFA in blue); 0.5 % MC (4. MC in light blue); 1 % VFA (5. VFA in orange); 1 % VC (6. VC in yellow). Scale is provided in cm.



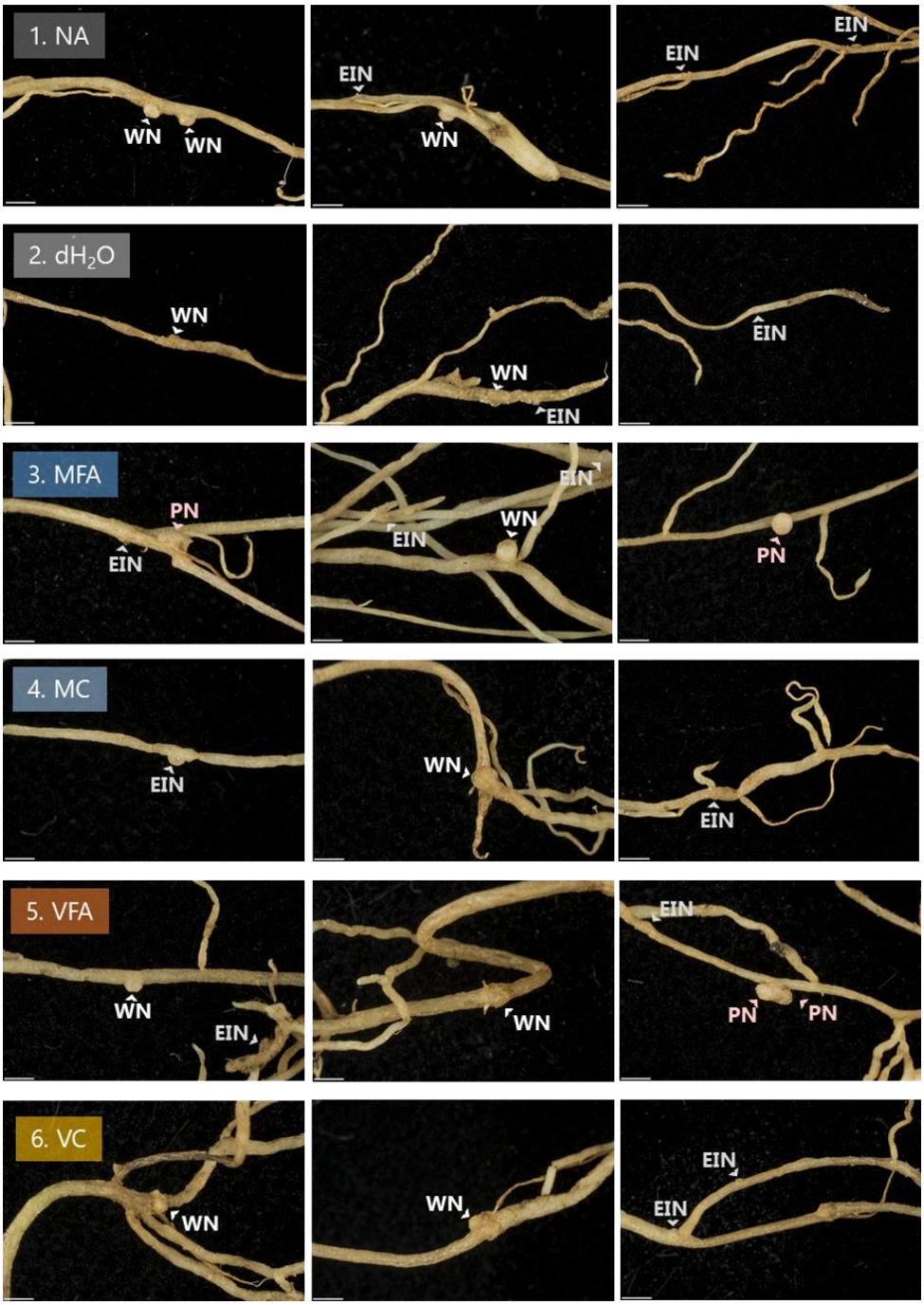
<u>Figure 5.5:</u> Vegetative biomass of *Medicago sativa* cultivars and *Lolium perenne* following treatment with fulvic acids or controls.

Treatments were applied to seedlings at 7 days post germination and vegetative biomass (dry weight in mg) were assessed at 21 days post treatment. Treatments were; no addition (NA in dark grey); deionised water (dH_2O in grey); 0.5 % MFA (MFA in blue); 0.5 % MC (MC in light blue); 1 % VFA (VFA in orange); 1 % VC (VC in yellow). Three cultivars of *M. sativa* were tested, cv. Daisy (a), Luzelle (b), and Gea (c). One cultivar of *L. perenne* was tested, cv. AberMagic (d). Individual seedling biomass was measured for three independent experiments, as shown in black data points (Exp. 1 = circles, Exp. 2 = triangles, Exp. 3 = squares). Box plots show variation across experiments. Multiple comparisons between treatments were conducted using a one-way ANOVA Tukey test shown with letter, with significance level of 0.05.

5.2.4 Fulvic acid application caused an increased number of pink nodules in *Medicago sativa*

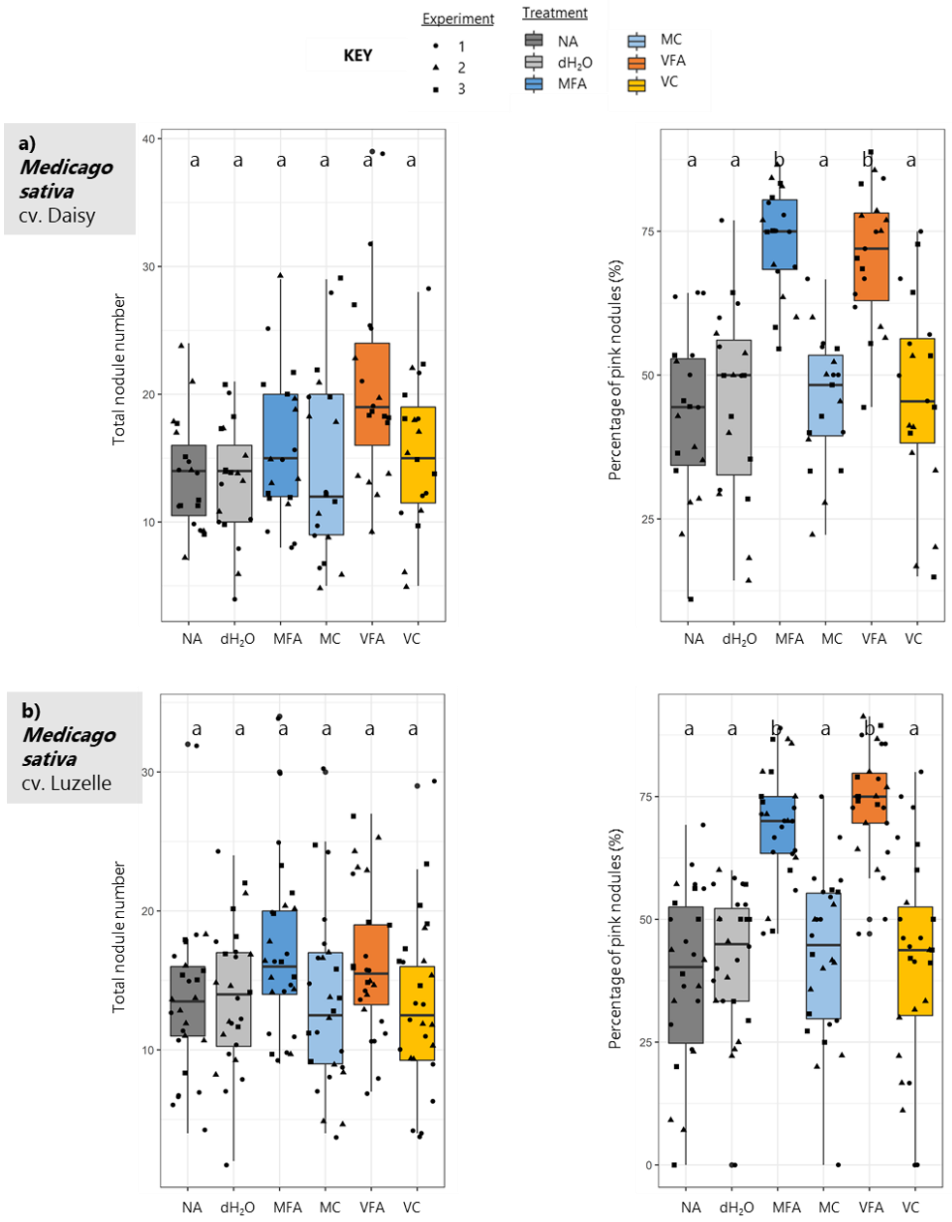
As *M. sativa* is a legume, and yield is known to be affected by the amount of nodulation in roots, the number of nodules were investigated in cv. Daisy and Luzelle. This count was done following application of MFA or VFA, or their controls. Figure 5.6 shows a representative visual scoring of nodules for each treatment. All treatments had plants which nodulated to varying degrees. Some roots had only early stage initiating nodules (EIN), whereas others had established white nodules (WN), or mature pink nodules (PN). No late stage nodules were found at this 21 day timepoint, which would be greyish green or brown in colour. EIN were not included in nodule counts as these may have never matured. Both MFA and VFA had visually more PN of large, mature size at 21 days than all other treatments. Total counts across three blinded experiments can be seen in Figure 5.7, alongside percentage of pink nodules. In other words, nodule numbers were counted without knowing the treatments applied. Again, MFA and VFA have more PN compared to all other controls, even if the total nodule number was only slightly increased. This nodulation phenotype is significant in both cultivars for both FAs, (see ANOVA letters in Figure 5.7, with significance level of 0.05).

The pink colour of large PNs is caused by the presence of leghaemoglobin, indicative that bacteria are actively N-fixing *Rhizobium* within the nodule. Therefore, it is possible that this increased rate of N-fixation caused by FA treatment may be the cause of increased vegetative growth. This could mean that FAs do not affect only the plant, but may also influence the N-fixing bacteria such as *Sinorhizobium*. More investigation is needed to identify whether the increased vegetative growth phenotype of FA treatment is found in the absence of *Rhizobium*.



<u>Figure 5.6:</u> *Medicago sativa* cv. Daisy nodules following treatment with fulvic acids (MFA or VFA) or controls.

Treatments were applied to seedlings at 7 days post germination and photographs above were taken at 21 days post treatment. Treatments were; no addition (1. NA in dark grey); deionised water (2. dH_2O in grey); 0.5 % MFA (3. MFA in blue); 0.5 % MC (4. MC in light blue); 1 % VFA (5. VFA in orange); 1 % VC (6. VC in yellow). Nodules are indicated as either early initiating nodules (EIN), white nodules (WN), or pink nodules (PN). Only white and pink nodules were counted as true nodules in for this analysis. Scale included is 1 mm.



<u>Figure 5.7:</u> Nodulation counts of two *Medicago sativa* cultivars following treatment with fulvic acids or controls.

Treatments were applied to seedlings at 7 days post germination and nodules counted at 21 days post treatment. Treatments were; no addition (NA in dark grey); deionised water (dH_2O in grey); 0.5 % MFA (MFA in blue); 0.5 % MC (MC in light blue); 1 % VFA (VFA in orange); 1 % VC (VC in yellow). Two cultivars of *M. sativa* were tested, cv. Daisy (a), cv. Luzelle (b). Individual seedling nodules were counted for three independent experiments, as shown in black data points (Exp. 1 = circles, Exp. 2 = triangles, Exp. 3 = squares). Box plots show variation across experiments. Multiple comparisons between treatments were conducted using a one-way ANOVA Tukey test shown with letters, with significance level of 0.05.

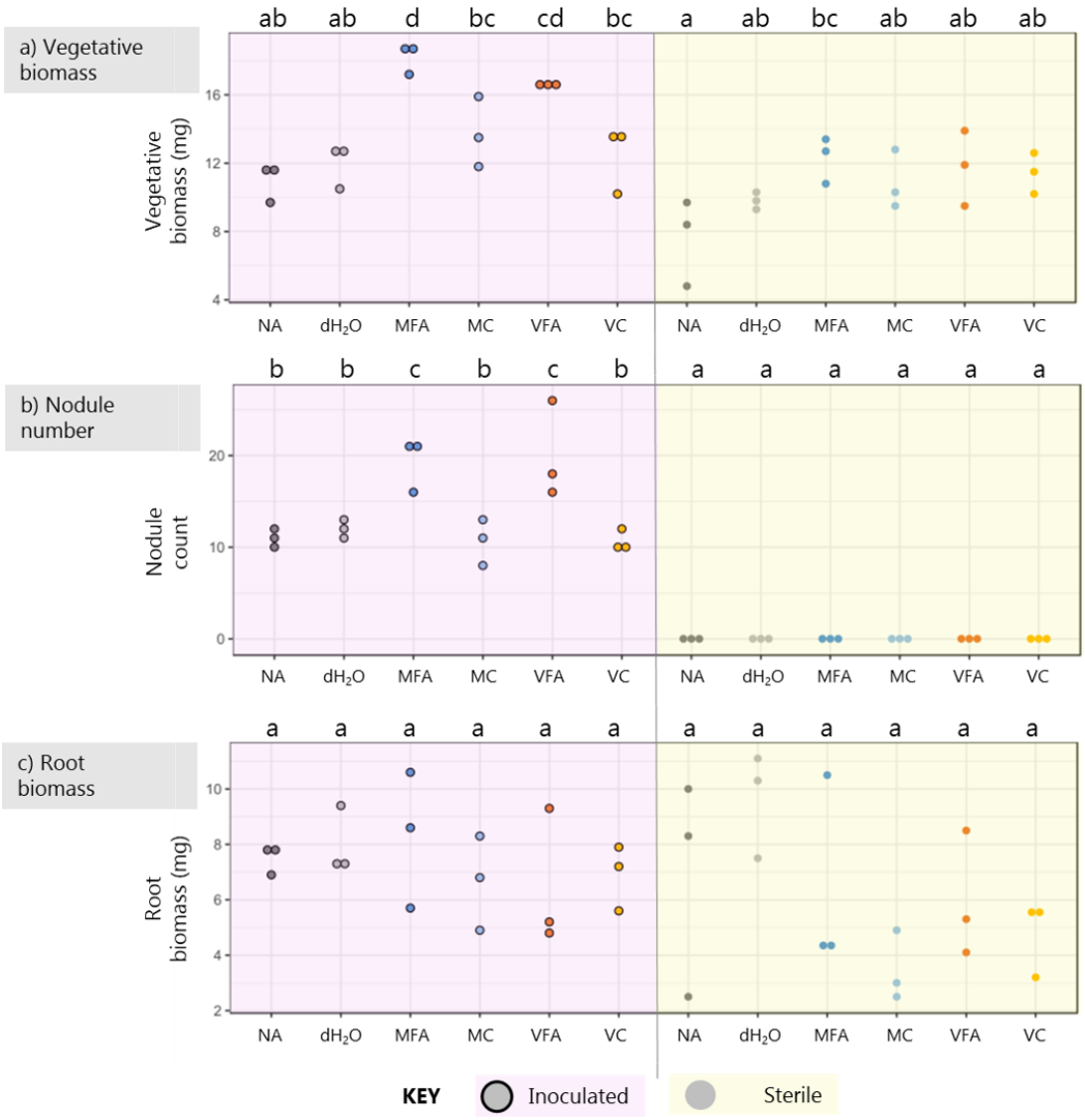
5.2.5 Vegetative growth phenotypes with Fulvic acid application is dependent on *Sinorhizobium* presence

Following on from the observation that FA treatment stimulated vegetative growth of *M. sativa,* it was decided to investigate this effect both in the presence and the absence of *Rhizobia*. To test this, the *Rhizobium Sinorhizobium meliloti* was selected *as M. sativa* is known to be its main host plant. Seedlings of similar sizes were transferred to sterile plates with treatment media and were either inoculated with *S. meliloti* or left sterile. After 21 days, plates were checked for nodulation and changes in both vegetative and root tissue biomass.

The results of this experiment can be found in the dot plots in Figure 5.8 for cv. Daisy only; results for cv. Luzelle and cv. Gea can be found in Figures C4 and C5, Appendix C respectively. Yellow plot areas with open sample dots are sterile plates, and light purple plot areas with closed dots indicate those plates inoculated. Vegetative biomass (a) was significantly increased with FA treatments compared to control when *S. meliloti* was present (inoculated plates), see ANOVA results indicated by letters with significance of 0.05. Average vegetative biomass of 15 seedlings with MFA treatment was 18.2 ± 0.9 mg compared to MC which was 13.7 ± 2.1 mg, and VFA treatment was 16.6 ± 0.2 mg compared to VC which was 12.4 ± 1.9 mg. MFA treated plants had an average vegetative growth increase of 167 %, and VFA an increase of 152 % relative to the no application (NA) control; cv. Luzelle and cv. Gea had average relative increases for MFA treatment of 142 % and 157 % respectively, and for VFA treatment of 146 % and 148 %.

Nodule number (b) was higher in both FA treatments for plants on inoculated plates only, (plants on sterile plates were unable to form nodules without *S. meliloti*). This increase in nodule number was strongly correlated with vegetative biomass (R^2 = 0.82). Again this regression was shown in cv. Luzelle and cv. Gea (R^2 = 0.78, R^2 = 0.76). This data was supported by glasshouse experiments in which more mature PN nodules were present in plants with FA treatment of VFA and MFA, (see Figures 5.6 and 5.7).

There was no significant difference in root biomass (c) with any treatment, regardless of *S. meliloti* presence or absence. Moreover, in sterile conditions only there were no significant differences in any measurement between FAs and their nutritional controls (see Figures 5.8, C4 and C5 in Appendix C). This shows the intrinsic link between nodulation and vegetative yield increase with FA treatment. The results suggest that investigations should be carried out on *S. meliloti* in isolation to see if FAs can affect its growth regardless from the plant.



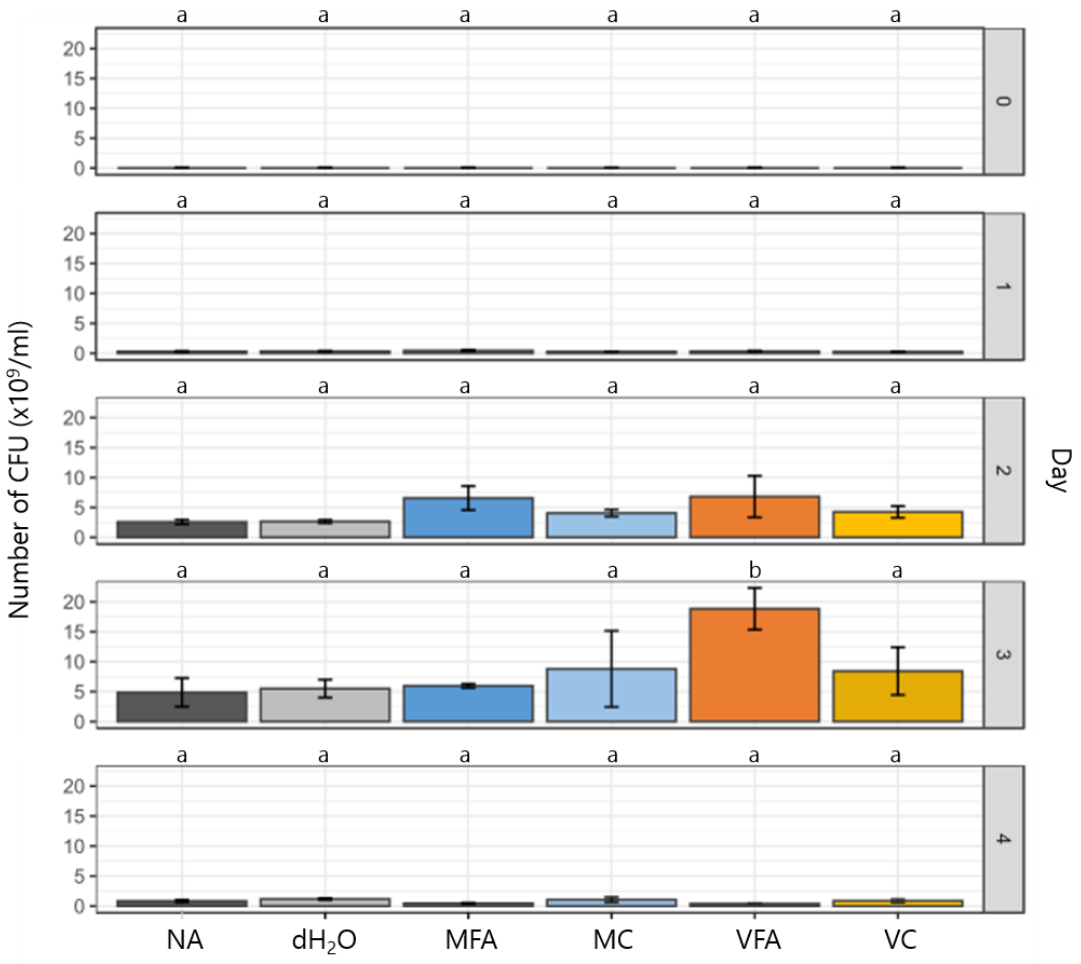
<u>Figure 5.8:</u> Vegetative and nodule phenotypes of *Medicago sativa* cv. Daisy following treatment with fulvic acids or controls, with or without inoculation of *Sinorhizobium meliloti.*

Two day old seedlings were transferred to media plates containing treatments as follows; no addition (NA in dark grey); deionised water (dH_2O in grey); 0.5 % MFA (MFA in blue); 0.5 % MC (MC in light blue); 1 % VFA (VFA in orange); 1 % VC (VC in yellow). Plates either remained sterile (open dots on yellow background) or inoculated with *Sinorhizobium meliloti* (closed dots on purple). At 21 days their nodule numbers were counted, and biomass determined for both vegetative tissue and full root tissue. Five seedlings were measured for each treatment condition, and total measurements for three independent experiments are shown in charts. Multiple comparisons were conducted using a one-way ANOVA Tukey test shown with letters, with significance level of 0.05.

5.2.5 *Sinorhizobium* growth is affected by fulvic acids

To determine if FAs can affect growth of *S. meliloti* in the absence of plants, the growth of the bacteria were tested in liquid culture using the standard microbial techniques of colony forming unit (CFU) counts. Cultures were treated with FAs or controls, and inoculated with *S. meliloti*, and the cell density tested over 4 days. An example of CFU counts can be found in Figure C6, Appendix C and the summary results are shown below in Figure 5.9.

There was no effect of FAs on culture cell density at 0 – 1 days, when the microbial population is moving from lag phase to exponential phase of growth. At 2 days both FA cultures had a higher cell density than their controls, with MFA having 6.56×10^9 compared to MC with 4.07×10^9 and VFA having 6.81×10^9 compared to VC with 4.26×10^9. By 3 days MFA culture cell density did not differ from controls, but VFA had a significantly higher cell density of 1.88×10^{10}. It is possible that MFA was also higher than controls at a timepoint between 2 – 3 days, but this was not measured. At 4 days all cultures were in the microbial death phase as cell density rapidly declines. These results, particularly for VFA, show adding FAs can increase growth of *S. meliloti* in isolated liquid culture.



<u>Figure 5.9:</u> Growth effects of fulvic acid on the growth of *Sinorhizobium meliloti* in TY medium, compared to controls.

TY cultures containing treatments as follows were inoculated with *Sinorhizobium meliloti*; no addition (NA in dark grey); deionised water (dH_2O in grey); 0.5 % MFA (MFA in blue); 0.5 % MC (MC in light blue); 1 % VFA (VFA in orange); 1 % VC (VC in yellow). Average colony forming unit (CFU) counts were obtained from triplicate samples on 0 - 4 days of incubation with shaking 220 rpm at 28 °C. Average counts for three separate experiments were calculated and shown above with standard deviation. Multiple comparisons between treatments were conducted using a one-way ANOVA Tukey test shown with letters.

5.2.6 Soil microbial population structures are affected by fulvic acid

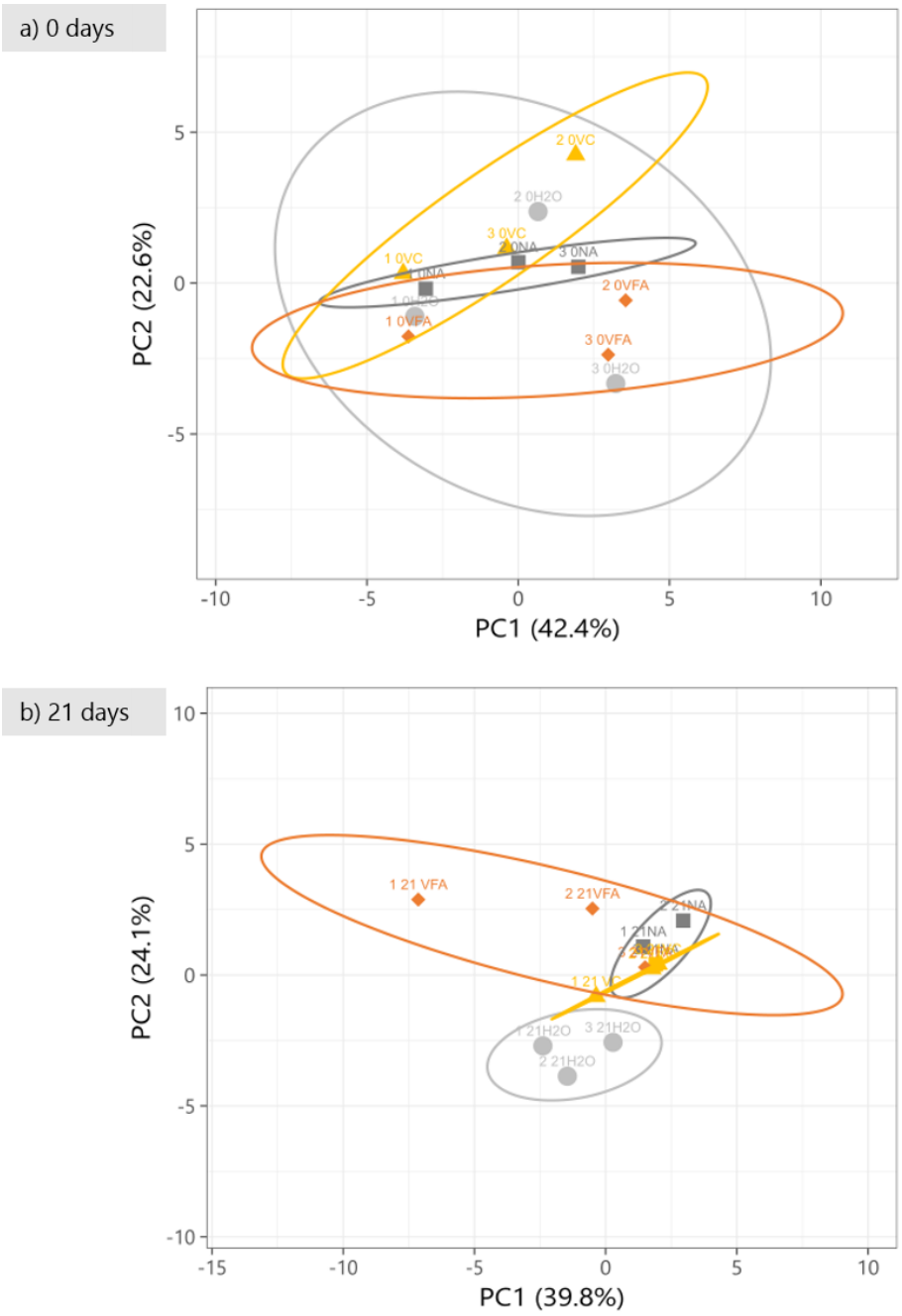
To test if soil microbes are affected by FAs in soil, a screen of changes in population was performed. Soil in which *M. sativa* was cultivated in glasshouse was treated with VFA, a VC control, a dH_2O control, or with no addition (NA) apart from a standard watering regime. Soil samples were then extracted to compare the relative phospholipid fatty acids (PLFAs) within them, both at day 0 and day 21 post treatment (see Chapter 2, section 2.3.14 for details). These PLFAs underwent transesterification to produce fatty acid methyl esters (FAMEs) Each FAME acts as a biomarker and corresponds to a microbial type, as developed in many studies throughout the past decades [665-669; 166; 670-672; 165]; the FAME biomarker assignments from Quezada *et al*., (2007) [166] were used in this study. The individual FAME relative amounts can be found in Appendix C in Figure C7, and groupings according to biomarker assignments are found in Figure C8.

It is possible to determine if the general microbial community composition has changed following a soil treatment by comparing principal component analysis (PCA) of extracted FAMEs between two timepoints. PCA plots provide statistical analysis of observations to convert variables into values to assess their correlation. In the case of FAME biomarkers, the plots correspond to how correlated the structure individual experiment treatments samples are to each other compared to other treatments. Figure 5.10 shows the results of this technique for VFA compared to controls.

At 0 days (Figure 5.10 a), treatments show overlap in their soil microbial structure, with all treatments having large variation across experiments. The variation in community data is 65 %, and this variation is shared across all treatments. At 21 days (Figure 5.10 b) variation has dramatically reduced for NA, dH_2O and VC as each PCA plot is reduced in size. It can be seen that dH_2O has diverged from other plots, but still overlaps with the other control treatments, suggesting the community composition still matches that of NA and VC. The VFA plot remains significantly larger and

skewed to the left of plot area, suggesting VFA treated soil is able to support a slightly different microbial population than other treatments.

This difference in VFA treatment PCA associated with FAME biomarker assignments is due to two main factors, (see Figure C8). From day 0 to day 21 for the VFA treatment, the relative amounts of gram-negative bacteria increased by ~ 4.09 %, whilst relative amounts of commonly shared FAMEs decreased by ~ 5.19 %. Moreover the relative amount of fungi increased the most when compared to other treatments at 0.72 %. The increase in relative gram-negative bacteria numbers is most interesting as this includes *Sinorhizobium* species. This means that the gram-negative bacteria proliferation matches the FA induced cell density increase found for the *M. sativa* symbiont *S. meliloti*, shown in Figure 5.9. More validation of this idea is needed, correlating FAME soil biomarkers for gram-negative bacteria with the *S. meliloti* increase in liquid culture following VFA treatment. This can be tested by quantifying *S. meliloti* DNA from the soil after VFA treatment.

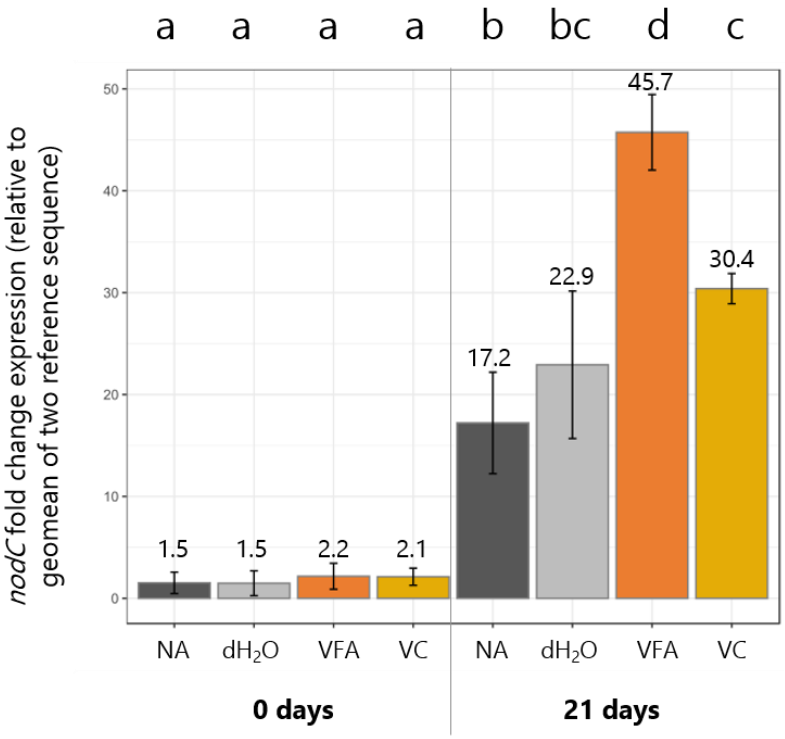


<u>Figure 5.10:</u> Two-dimensional principal component analysis (PCA) of fatty acid methyl esters (FAMEs) relative contents from soil treated with fulvic acid or controls.

Soil from *Medicago sativa* assays with fulvic acid applications (NA in dark grey; dH_2O in grey; VFA in orange; VC in yellow) was extracted for PLFA content at 0 days (a) and 21 days (b), for three separate experiments (indicated as 1, 2, and 3), and converted into FAMEs. Individual FAME relative abundance (in % of total FAMEs) was calculated from total FAME biomass (nmol g^{-1} of dry soil). FAMEs were associated with particular organisms [166], and PCA plots calculated, variation of community data for 0 day and 21 days was 65 % and 63.9 % respectively.

5.2.7 Quantification of DNA from soil substrate confirms fulvic acid increases *Sinorhizobium* numbers

To test if the *S. meliloti* population increased in soil treated with FA, total DNA was extracted from soil from the same origin as that used in 5.2.6. DNA was quantified for relative amounts of *S. meliloti* specific gene *nodC* [170] using real-time PCR alongside two reference gene sets 799/1391 [172; 173; 171] and Eub338/518 [175; 176; 174]. Results are found in Figure 5.11. At 0 days all soil has the same level of *S. meliloti* detection regardless of treatment, shown in the fold change of *nodC* being around 1.5 – 2.2 fold change expression for all treatments. At 21 days all treated soils have increased in *nodC* expression, as *S. meliloti* populations have multiplied over time with growth of *M. sativa*. However, VFA shows a significantly increased *nodC* change in expression compared to other treatments at 45.7. This change is indicative of an increase of *S. meliloti* number in this treatment, which provides more data that VFA can increase the growth of *S. meliloti*. This proliferation may be associated with increased nodulation in *M. sativa* due to the plants increased growth following VFA treatment, as shown in Figure 5.5.



<u>Figure 5.11:</u> Quantification of *Sinorhizobium meliloti* DNA using *nodC* gene from soil treated with fulvic acid or controls.

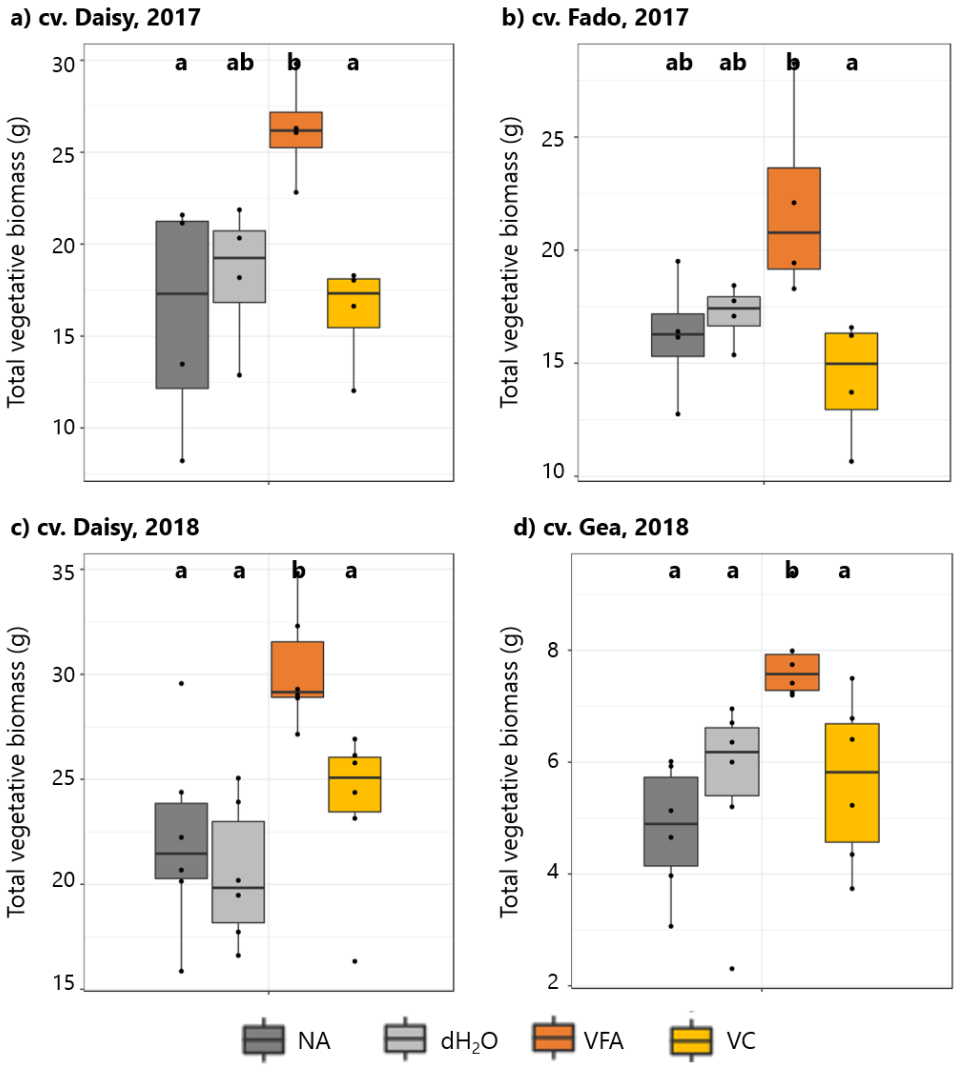
Soil from *Medicago sativa* assays with fulvic acid applications (NA in dark grey; dH_2O in grey; VFA in orange; VC in yellow) was extracted for PLFA content at 0 days and 21 days, for three separate experiments. DNA was extracted from soil samples and geometric expression of *nodC* was calculated using real-time PCR, with fold change relative to two reference sequences, indicated above each bar. Error bars are standard deviation of relative expression of experiments. Multiple comparisons were conducted using a one-way ANOVA Tukey test shown with letters, with significance level of 0.05.

5.2.8 Fulvic acid vegetative growth effect on *Medicago sativa* was recorded in independent field trials, with no change to nutritional content

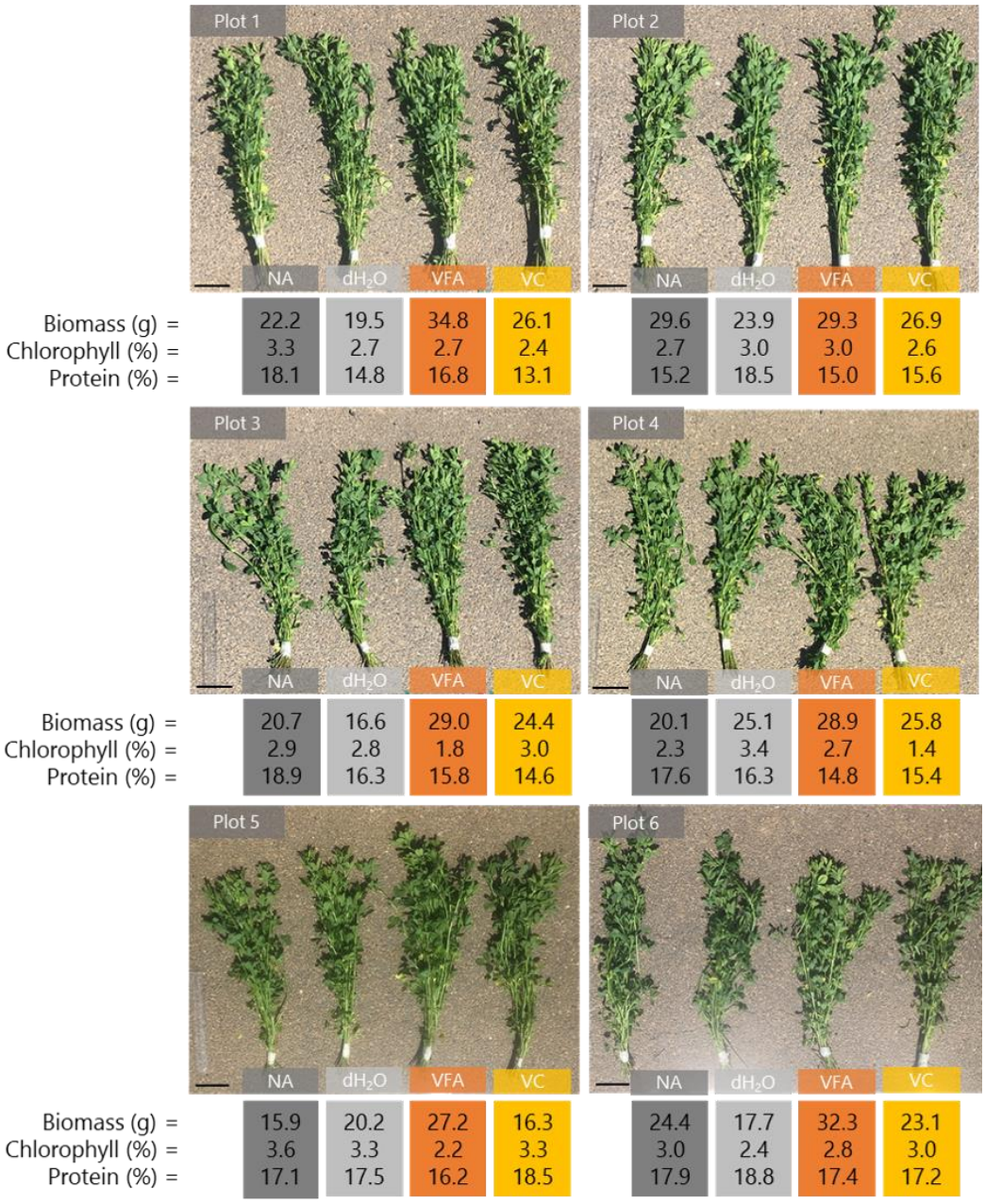
It is necessary to assess whether FA treatment can give a vegetative growth increase in *M. sativa* in the field as all previous experiments have been conducted in glasshouse conditions. Over 2017 and 2018, trials were conducted at three sites, all of which are designated forage crop cultivation farms. Field plot plans and maps of the trials are found in Figure C9, Appendix C. For each trial, plots were treated with either VFA, the control solution VC, a dH_2O control, or with no addition (NA) at early establishment of *M. sativa* (April – June). Plots were grown in accordance with site usual management practices, with treatment applications the only difference.

Prior to the first harvest of the season (May – July), vegetative biomass was measured for treatment plots, shown in Figure 5.12 a)-d). Although different cultivars were treated on the various sites, VFA increased vegetative biomass in all trials. Biomass increased with the VFA treatment for vegetative tissues, in both shoot and leaf tissues. This increased growth compared to NA was 135 – 165 %, which is similar to the boost found in plate assays of 146 – 152 %, (see Figure 5.8), and slightly lower than measurements from glasshouse experiments of 167 – 185 %, (see Figure 5.5).

Nutritional content of *M. sativa* from each treatment plot was also assessed for the 2018 trials. Figure 5.13 shows samples from Blankney Estates Ltd. trial with total vegetative biomass measurements, along with chlorophyll and total protein levels of each. There was a significant difference in vegetative biomass, but no difference in either chlorophyll or total protein content for any treatment. This was also found for A Poucher and Sons Ltd.; for NA, dH_2O, VFA, and VC plots the average chlorophyll and total protein content was as follows; 2.91 and 17.87 %; 2.61 and 17.96 %; 2.83 and 18.21 %; 2.63 and 17.97 %. This data provides evidence that the effect of VFA is on increasing biomass yield with no change in nutritional content. It is concluded that the VFA stimulatory effects found in the laboratory are applicable in the field.



<u>Figure 5.12:</u> Vegetative biomass of *Medicago sativa* cultivars in independent field trials following treatment with a fulvic acid or controls. Treatments were applied to field plots at beginning of establishment and vegetative yields were assessed before 1st cut of growing season; an area of 625 cm^2 was sampled and total vegetative tissue dried for biomass (in g). Treatments were; no addition (NA in dark grey); deionised water (dH_2O in grey); 1 % VFA (VFA in orange); and 1 % VC (VC in yellow). Four trials were run over two years. In 2017 trials were performed at a)-b) Dengie Crops Ltd. (Southminster, Essex) with four plots per treatment of cv. Daisy and Fado. In 2018 the trials were at c) Blankney Estates Ltd. (Blankney, Lincolnshire) and d) A Poucher and Sons (Bardney Dairies) Ltd. (Market Rasan, Lincolnshire) with six plots per treatment of cv. Daisy and Gea respectively. Individual samples are shown with black dot points, with boxplots for each treatment in trials. Multiple comparisons were conducted using a one-way ANOVA Tukey test shown with letters, with significance level of 0.05.



<u>Figure 5.13:</u> Vegetative tissue of *Medicago sativa* of first cut following fulvic acid treatment compared to a control, from field trial plots.

Treatments were applied to field plots at beginning of establishment and vegetative yields were assessed before 1st cut of growing season; an area of 625 cm^2 was sampled and total vegetative tissue dried for biomass (in g), with value indicated. Treatments were; no addition (NA in dark grey); deionised water (dH_2O in grey); 1 % VFA (VFA in orange); and 1 % VC (VC in yellow). Samples are from 2018 trial plots at Blankney Estates Ltd. (Blankney, Lincolnshire) for cv. Daisy. Samples were duplicated for chlorophyll and protein contents, these are provided as total dry weight %. Multiple comparisons were conducted using a one-way ANOVA Tukey test with VFA having a significant increase in biomass samples only.

5.3 DISCUSSION

5.3.1 *Medicago sativa* vegetative yield can be increased by adding fulvic acid into crop management practices

This study demonstrates how applying a low concentration of FA to *M. sativa* can increase its vegetative yield, as shown by growth on plates (Figure 5.8), in glasshouse conditions (Figures 5.1, 5.2, 5.4, and 5.5), and in the field (Figures 5.12 and 5.13). This data builds on existing indications of a yield effect of HS in *M. sativa* and other forage legumes [645; 647; 648]. Grass species were not increased in vegetative biomass in this project, see Figures 5.1 and 5.2. As a relatively minor addition to current management practice, applying FA to forage legumes may be an exceedingly cost-effective technique in improving yields. As such, an important crop in the UK and globally, any small intervention to increase its yield is of significant economic importance. However, as *M. sativa* is not the only important temperate forage legume, with *Trifolium repens* and *Trifolium pratense* also being highly utilised, it is important that more analysis of FAs effect on other species is assessed. This should be performed in the field where possible, with strict adherence to negating any purely nutritional effects of treatments where possible by using appropriate control solutions as in this project.

5.3.2 The vegetative growth increase is associated with microbial changes including *Sinorhizobium* populations

This study has provided evidence that the vegetative yield in *M. sativa* following FA treatment is associated with changes in microbial populations. This is shown by increases in nodulation and in numbers of *Sinorhizobium* species especially (Figures 5.6 – 5.11), but also potentially with changes in other microorganism populations (Figure 5.10). Isothiazolone was found in low levels in VFA but changes in microbial growth were still detected. This means some additions to commercial products may not always have

the desired effect in application due to low concentrations when diluted. This again confirms a need for thorough investigation of commercial treatments including FAs.

The data in this project demonstrates that FAs may increase growth in plants through stimulating the growth of *S. meliloti*, or through affecting both plant and microbe together. It is necessary to see whether FA is able to affect the growth of other soil microbial populations as many organisms can increase plant vegetative yield, such as other *Rhizobium* species but also species of *Streptomyces*, *Bacillus* and arbuscular mycorrhiza fungi [604]. This can be used to test if the growth effect of FA is affecting vegetative biomass by increasing nodulation. Alternatively, FA may increase vegetative biomass in plants which then has an effect on increasing nodulation.

The effect of FAs on increasing microbial cell growth, without plant interaction agrees with other published studies. It has been previously observed that HS are able to increase growth of *Bradyrhizobium liaoningense* in liquid culture [673] and increase growth of general microbial populations in soil microbial cells [674]. However, a study of the yeast *Candida utilis* found no change in growth with HS, so this response may be taxa dependent [675].

An improved symbiotic association of crops with microbes is important in the light of the current emphasis to grow more perennial and annual leguminous crops globally, due to their N-fixing activity [676-679; 578]. The fixing of atmospheric N_2 in legume/grass pastures is estimated to range from 13 to 682 kg N ha^{-1} yr^{-1} [680]. *M. sativa* has been estimated to have a fixation rate of up to 350 kg N ha^{-1} yr^{-1}, this provides an N fixation rate of 0.021 x DM + 16.9 for *M. sativa* (R^2 = 0.91) [681], despite large differences in the N status of the soil through fertiliser use and geographic location. Such N fixing calculations are important when accounting for tissue N allocation and the soil residual N available for future crops. For *M. sativa* tissue allocations of total plant N is approximately 50 % in shoot, 45 % root, and 5 % nodule [682; 280]. Studies in which legume stubble and root are left in ground post-harvest have shown that a considerable proportion of biomass and fixed N can provide substantial N fertilisation

for future crop rotations [683]. This means that the increased vegetative growth in a cultivated legume as shown in this study could have implications for the yields of future crops.

More elucidation of FAs effect on both plant and microbial communities is required. For future plant-focussed research projects, an assessment of potential N-fixing rate of symbiotic bacteria caused by changes from FA treatment could use the acetylene reduction assay or similar microbial bio-assays [684; 685; 682; 686]. An analysis of the host plant's transcriptional changes that occur upon application may be interesting to provide information for the mode of action of plant vegetative increase following the treatment; such an analysis will be discussed in the following chapter.

In addition to studying changes in plant growth, studies of the soil could determine how FA may affect microbial communities. Studies could use techniques in the field to determine changes in microbial biomass C or soil enzymatic activity assays to determine potential changes in soil function due to the illustrated changes HS may have on soil structure [625; 614; 615; 682; 632; 621; 607; 648]. Using 16S rRNA sequencing could also be used to investigate in more detail the changes in abundance between taxa [687].

5.3.3 Fulvic acids are complex chemicals and require more work to define their mode of action, including field applications

It may be argued that the most notable advancement of this study is the use of chemical analysis of FAs to develop controls for nutritional content of treatments. These controls have been missing from previous studies, and therefore makes accurate interpretation of the data impossible. A lack of controls in previous studies may be why many have surmised there is general hormone-like response by plants upon HS application [653; 654; 643; 655; 121], as such changes in root architecture and nutrient uptake may be a fertiliser effect only. This studies provides evidence that the response is not only a nutritional response, shown in vegetative biomass increases of VFA and MFA application compared to VC and MC respectively,

shown in Figures 5.4, 5.5, 5.8, 5.12, and 5.13. Nevertheless more work is required to begin untangling the mode of action.

This is particularly true when looking at the chemical analysis of both FAs in this study alone; the FAs were sourced from different locations and showed they had largely differing contents, (see Figures 5.3, C2 and C3, Appendix C, and Tables 2.8 and 2.9). There has been a push for standardisation of HS analysis including separation of C-containing groups recently [609; 629]. However, until a standard analysis of HS is used to develop nutritional controls in plant or microbial studies, then no real advances can be made into how these treatments may promote effects.

In addition to this, it is also necessary for more studies to investigate FA in other contexts. Many studies have relied on only glasshouse and pot experiments without field testing, which may affect the abundance and diversity of soil microbial communities [688]. More analysis of soil chemical and physical parameters could also be explored, to conclude if HS has a soil conditioner effect [614]. It should be noted that such an examination may conclude that HS are not biostimulants if they also have such effects [120].

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Chapter 6: Transcriptome analysis shows preferential enrichment of nodulation regulation and signalling-related genes in *Medicago sativa* following fulvic acid application

6.1 INTRODUCTION

6.1.1 Using RNA-seq to identify transcriptional changes in plants

In the past decade, RNA-sequencing (RNA-seq) has changed from a complex and challenging tool in the early-stage of conventional use [689; 690] to a critical technique used in many studies that wish to investigate how phenotypic changes occur in specific conditions [691]. Changes in the pattern of total RNA expression can reveal the signals and metabolic changes that underpin a phenotype. As a tool, RNA-seq is incredibly robust and sensitive [692-695] and provides a wealth of data for a researcher, be it a specific line analysis, treatment investigation, or stress assay.

The increased prevalence of using RNA-seq is for multiple reasons. As with all technologies, the cost has significantly reduced over the past ten years, in both financial terms and through decreased time needed to carry it out with recently improved protocols [696]. The wealth of bioinformatic support has improved with early-career researchers developing computational skills for analysis, with many universities and institutes employing dedicated bioinformations to aid this [697], although not without problems in resource and credit allocations [698]. A vast array of programmes has been developed to execute this high-throughput method, from read mapping to transcriptome reconstruction to expression quantification [699; 691]. Furthermore, these are combined with improved reference genomes of many model species, providing substantial in silico evidence which can be coupled with experimental work to build new biological hypotheses. Model legumes have been analysed for transcriptomic changes, including *Medicago truncatula* [700-706; 178; 707; 708] and *Lotus japonicus* [709; 354; 710; 711; 708].

RNA-seq has been used in many non-model plants using *de novo* analysis or mapping to closely related reference genomes. These include the legume crops chickpea [712-714], pea [715-717], *Medicago lupulina* [717], lentil [718], soybean [719], lupin [720], clover [347], and mung beans [721]. RNA-seq has been used successfully in many studies for

Medicago sativa for comparative line studies [722-724] and stress responses [725-730].

6.1.2 The effect of Fulvic acid on *Medicago sativa* transcriptome in both shoot and root tissue

In Chapter 5, a vegetative biomass yield increase in *M. sativa* was shown following fulvic acid (VFA) treatment. In previous publications the reasons for this are unknown and studies have suggested wide-ranging modes of action for this biostimulant. Therefore, it was necessary to investigate the transcriptional changes that may have occurred in the shoot and root tissue using RNA-seq analysis. As *M. sativa* is a legume, yields are strongly associated with the symbiotic N-fixing symbiotic bacteria termed *Rhizobium* [731].

Deriving from 'rhizo' meaning root [732], the rhizosphere is the root/soil interface where a high microbe population can be found. The rhizosphere is generally defined as the area of soil that is influenced by the activity of a root. The rhizosphere around an expanding root establishes gradients between the plant and surrounding soil that are important for nutrient and water uptake. The secretion of root exudates and subsequent symbiotic relationships with rhizobial microbes that are attracted by such secretions [733; 734] are important for nutrient delivery in the rhizosphere. Organisms which interact with the plant roots include *Rhizobium* [731; 735], but can also include members of other microorganism families, including a high proportion of fungi, protozoa, and nematodes [736; 737]. The legumes are a family of plants, which include *M. sativa,* that can form a symbiotic relationship with *Rhizobium*, shown in about 90% of the *Leguminosae* family [738]. In terms of legume and symbiotic *Rhizobium*, each must recognize each other to establish such a relationship. This is mediated through an infection of the bacteria into the root via the endorhizosphere or ectorhizosphere, with symbiosis being viewed as mutualistic, communalistic, or pathogenic. Symbiosis then proceeds via the development of special structures termed nodules on the root through rhizobial infection [739; 740].

Due to the dependence of *M. sativa* vegetative biomass yield on N supply through rhizosphere interactions (see Chapter 3), root tissue will be analysed for transcriptional changes caused by VFA using RNA-seq.

6.1.3 *Rhizobium*/legume nodulation initiation

For a symbiosis to occur between the legume and a *Rhizobium* they must first communicate with each other to allow infection into the host [731; 735]. This is caused via the pattern of cytoskeletal rearrangement of cells caused by calcium influx [741], root-hair curl around the bacteria to form an infection pocket, and infection thread formation by the *Rhizobium*, all of which lead to infection and colonization [740; 738]. Transcriptomic data in legumes supports the idea that plant defence must be suppressed by *Rhizobium* at thread formation to successfully reach the nodule and be successful [709; 701; 742]. This ultimately provides the transfer of carbon resources from plant to bacteria and nitrogen resources from bacteria to plant within nodules [743].

Plants use chemical signals to attract *Rhizobium*, primarily via flavonoids, which initiate crosstalk and symbiosis between the organisms [744]. Flavonoids have been shown to be specific for certain *Rhizobium*, for example in soybean the secretion of daidzein and genistein induce *Bradyrhizobium japonicum*, whereas *Sinorhizobium meliloti* is inhibited by these and instead induced by the compound luteolin [745]. These chemicals are then perceived by the *Rhizobium* as aglycones, which lead to the induction of *nod* genes by their interaction with the LsyR-type nod gene regulator [746; 744; 747]. The *nod* gene family is large and diverse, although with a high degree of conservation within their promoter region. Usually, as in the case of *NodV*, the flavonoid signal phosphorylates the NodV protein which in turn activates another nod gene, such as *NodW* [748].

These Nod factors can then be recognized by the LysM-type receptors of the host plant [749-754], and subsequently nodule development and bacterial infection can occur [755]. The symbiosis that results is dependent on the degree to which the plant allocates carbon resources to the

Rhizobium, and vice versa, the extent that the *Rhizobium* gives N to its host. It is also hypothesized that local immunosuppression of the host root by the *Rhizobium* is essential to set up the symbiosis. This is thought to be achieved by the nod factors suppressing the normal immunity of the legume by inducing the microbe/pathogen-associated molecular pattern (MAMP/PAMP) [756; 757], which in turn affects the plant's pathogen-/pattern-recognition receptors (PRRs). PRRs are able to recognize molecules which are microbe-associated [758-760]. Moreover, other factors that influence the formation of nodules in the legume/*Rhizobium* symbiosis include DNA-binding transcription factors such as the membrane-localized GmbHLHm1 in soybean. Loss-of-function mutants of GmbHLHm1 showed many defective phenotypes such as reduced levels of leghemoglobin, smaller-infection zone of the *Rhizobium*, reduced nodule number and fresh weight [761].

Mutualistic relationships between plants and *Rhizobium* generally involve this sharing of resources, enabling both organisms to gain from their interactions with each other. A common example is that of soybean and *Bradyrhozbium* [762], and a widely studied example of a non-legume symbiosis is the relationship between the cyanobacteria, *Anabaena*, and its host the aquatic fern, *Azolla* [763]. *Medicago sativa* forms preferential symbiosis with *Sinorhizobium meliloti* [764-768].

Some *Rhizobium* can form a symbiotic relationship with a large variety of legumes. For example, the *Rhizobium* strain NGR234 has been shown to nodulate over 200 species and even affect some non-legume species, which enables mutualistic interaction between both organisms as described above [769]. Moreover, there are extra positive interactions found from these mutualisms; for the plant this can include an increase in abiotic and biotic stress tolerance and its ability to grow in soil where other microbes produce toxic compounds that the mutualistic *Rhizobium* are able to degrade [770; 744]. Furthermore, the production of biofilm layers can also protect against pathogens [771].

6.1.4 Aims of this chapter: RNA-seq analysis in *Medicago sativa* to investigate changes in transcription following fulvic acid application

This chapter describes work investigating the transcriptional changes that occur in *M. sativa* upon FA treatment. This will help investigate the causes for the yield increase shown in Chapter 5. The commercial FA referred to as VFA was tested alongside a control solution called VC defined in 2.3.9 and 5.2.2. Following three days of treatment plant tissue was analysed using RNA-seq using shoot and root tissues separately. Transcriptome analysis was performed *de novo* and using related *M. truncatula* reference accessions.

The aim was to identify transcriptomic changes which occurred following VFA treatment in the plant. This can be used alongside the results in Chapter 5 to identify the FA mode of action for yield increases in legumes.

6.1.5 Materials and Methods

The methods used in this chapter are detailed in Chapter 2, in section '2.4 FULVIC ACID RNA-SEQUENCING OF MEDICAGO SATIVA'. Supplemental data is provided in Appendix D.

6.2 RESULTS

6.2.1 VFA induces differential expression of transcripts in *Medicago sativa* after 3 days of treatment

RNA was extracted from shoot and root tissues of *M. sativa* treated with either VFA or the nutritional control VC, on the day of treatment (day 0) or three days after the treatment (day 3). Samples were generated from three experiments and analysed using RNA-seq. Differentially expressed (DE) transcripts were analysed in shoot and root tissues. As *M. sativa* does not have a fully annotated sequenced genome, many studies have used *M. truncatula* genomes as a mapping reference. The references accessions A17 [702; 707] and R108 [178] show good alignment for studying transcripts (60 – 70 % for A17 and 75 – 85 % for R108), but *de novo* transcriptome assembly was performed to negate for bias in subsequent analysis. *De novo* transcriptome assembly was successful for building a scaffold (shown in Table 6.1 below) with similar alignment rates of all transcripts for A17 and R108 references. This was possible due to the high quality of samples used for sequencing, and shown in the sequencing quality checks in Table D2, Appendix D.

Multi-dimensional scaling (MDS) plots were generated to compare all transcripts in experimental replicates. These are provided in Figure 6.1, with both 2D and 3D plots for both shoot and root samples. The MDS plots demonstrate that the transcripts clustered for treatment and timepoints among samples. This is especially clear at day 0 where both VC and VFA were very similar in their clustering as blue and green plots, in both tissues. This might be expected for day 0 when any differences in transcripts will be through random chance and insignificant in later analysis. In both tissue types, both 2D and 3D plots show clearly that not only did day 0 VC and VFA cluster together (blue and green), but moreover day 3 VC and VFA clustered farther away with greater separation (orange and red), indicating developmental stage transcriptional changes. The 3D plots especially indicate in both tissue types that the day 3 VFA clustering

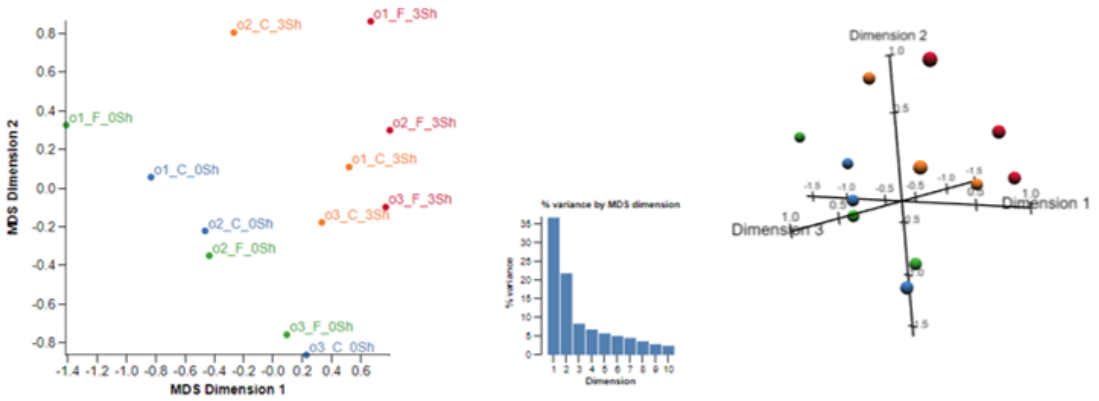
is more distant from the rest, suggesting more transcriptional variation from the other samples due to the treatment effect.

Total transcripts were analysed to find the significantly DE transcripts for each treatment between the two timepoints. The determination of significantly DE transcripts required use of both an absolute log fold change of 0.585 (1.5 x fold change), and a false-discovery rate (FDR) adjusted p-value (q-value) < 0.05, with removal of DE transcripts significantly expressed between treatments at day 0 to negate for false positives due to experimental variance. The number of up-regulated (+) and down-regulated (-) DE transcripts in shoots and roots of *M. sativa* following either VFA and/or VC treatment is shown in the Venn diagram below, Figure 6.2. This result shows that most DE transcripts for VFA treatment occurred in the root tissue. There were found to be 1705 upregulated and 241 downregulated DE transcripts in the root, and 140 upregulated and 209 downregulated DE transcripts in the shoot. This difference in DE transcript number is emphasised by differing sizes of the circles in the plot.

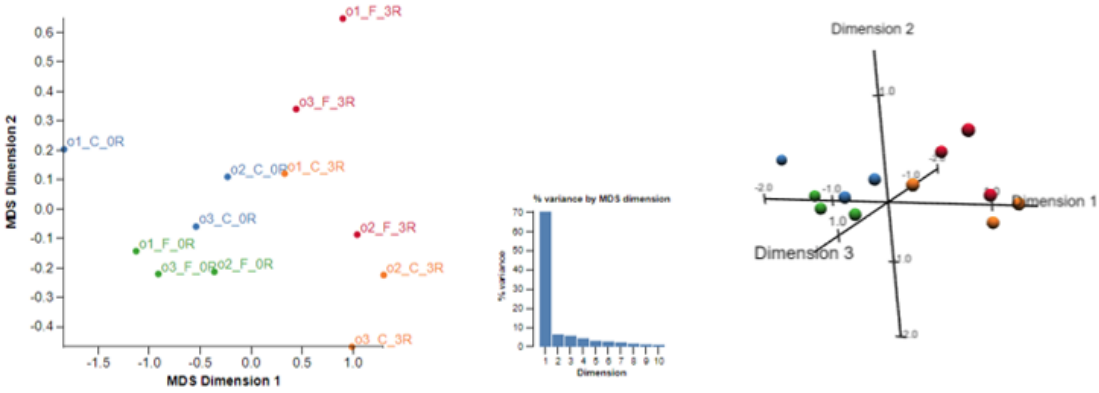
Table 6.1: Pseudoalignment summary of RNA-seq samples with *de novo* transcriptome assembly.

Treatment	Tissue	Time point	Rep	Reads	Reads pseudoaligned	% pseudoaligned
Control	Shoot	0	1	19731245	13433374	68.08
Control	Shoot	0	2	20017217	13281556	66.35
Control	Shoot	0	3	22060176	13759703	62.37
Control	Shoot	3	1	23125595	14603288	63.15
Control	Shoot	3	2	22410937	14604137	65.17
Control	Shoot	3	3	22566458	15036502	66.63
Fulvic	Shoot	0	1	21948373	15001567	68.35
Fulvic	Shoot	0	2	20015180	13166950	65.78
Fulvic	Shoot	0	3	19550309	12674614	64.83
Fulvic	Shoot	3	1	21916558	13940709	63.61
Fulvic	Shoot	3	2	20834945	13749556	65.99
Fulvic	Shoot	3	3	21961363	14221382	64.76
Control	Root	0	1	19192851	13111977	68.32
Control	Root	0	2	18832338	13193369	70.06
Control	Root	0	3	19868638	14429523	72.62
Control	Root	3	1	21818824	15337857	70.30
Control	Root	3	2	19940906	14437150	72.40
Control	Root	3	3	18121577	12993129	71.70
Fulvic	Root	0	1	21683448	15322780	70.67
Fulvic	Root	0	2	22756103	15965816	70.16
Fulvic	Root	0	3	20587135	14609678	70.97
Fulvic	Root	3	1	19970525	14133478	70.77
Fulvic	Root	3	2	21347709	15176499	71.09
Fulvic	Root	3	3	20853270	14884900	71.38
			Total	501111680	341069494	-
			Mean	20879653	14211228.92	68.15

a) Shoot tissue RNA-seq samples

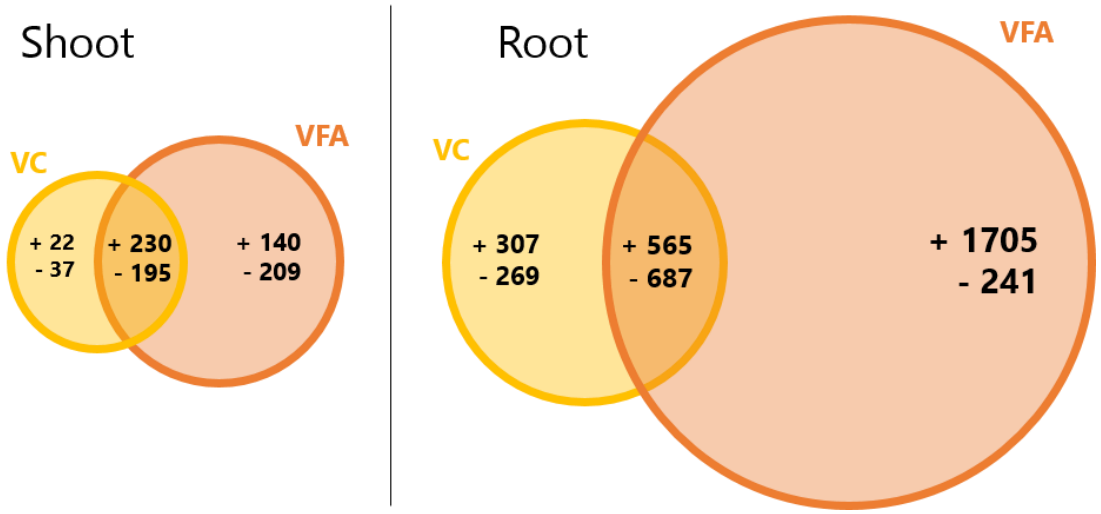


b) Root tissue RNA-seq samples



<u>Figure 6.1:</u> Multi-dimensional scaling (MDS) plots to compare all transcripts in experimental replicates clustered for treatment and timepoints.

Shoot (a) and root (b) data is shown separately, with the left-hand graphs generated using Degust [183] showing a 2D MDS plot of samples as follows; day 0 VC is blue; day 0 VFA is green; day 3 VC is orange; day 3 VFA is red. Bar charts are % variance of MDS plot. Right-hand graphs show a 3D MDS plot. In all cases MDS is generated with 200 transcripts.

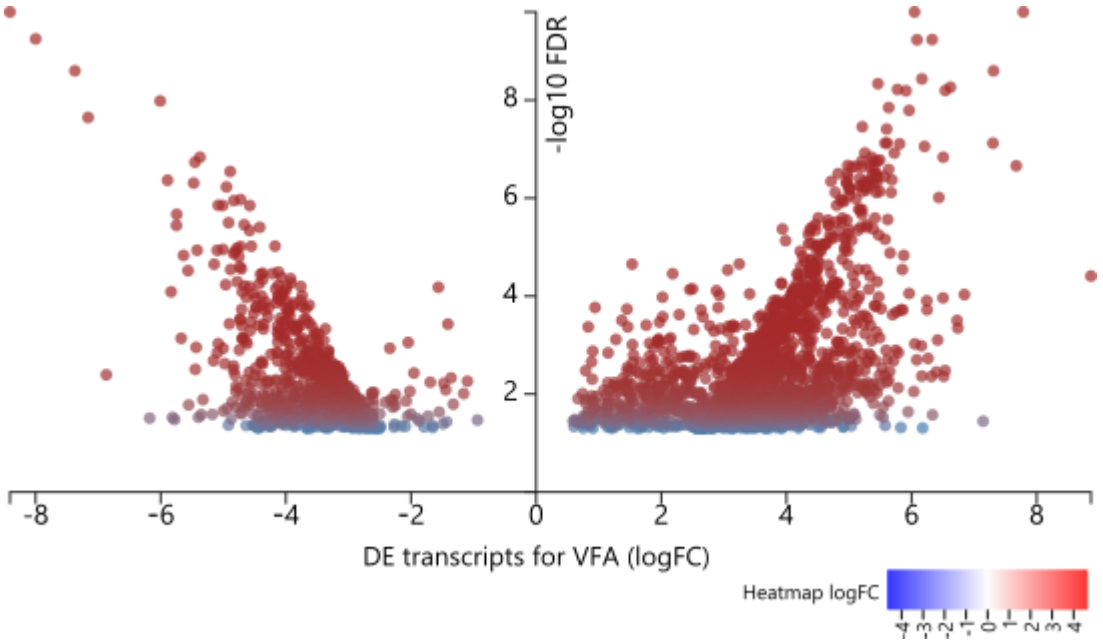


<u>Figure 6.2:</u> Differentially expressed transcripts in *Medicago sativa* shoot and root tissue with treatments of either fulvic acid (VFA, orange) or the control (VC, yellow).

RNA-seq was carried out on whole shoot and root RNA samples taken on day of treatment (day 0) or three days after treatment (day 3). Transcripts from *de novo* transcriptome assembly with both an absolute log fold change of 0.585 (1.5 x fold change) and a false-discovery rate (FDR) adjusted p-value (q-value) < 0.05 were considered as differentially expressed (DE); DE transcripts significantly expressed between treatments at day 0 were removed to negate for false positives due to experimental variance. The above Venn diagram shows upregulated (+) and downregulated (-) DE transcripts for both treatments between day 0 and day 3, including those which are shared (overlapping region).

6.2.2 Significant transcriptional changes occured in roots following VFA treatment

As the above analysis found that shoot and root tissue had DE transcripts due to VFA treatment, both tissue type samples were used in the subsequent analysis. However as shown in Figure 6.2, a far higher amount of expression changes were found in the root than the shoot. Therefore, a focus was placed on root samples as it is likely they would provide more evidence of VFA mode of action than shoot data. Below in Figure 6.3 is a volcano plot that indicates the statistically significant DE transcriptional changes that occured in the root tissue between day 0 and day 3 for VFA treatment. All significant DE transcripts for shoot and root tissues can be found in Appendix D Figure D2. Comparison of 0 VC to day 0 VFA, and 0 VC to day 3 VC, also determined some DE transcripts. These DE transcripts were removed from subsequent analysis of the VFA treatment to ensure only transcripts truly differentially expressed by plants undergoing the VFA treatment are functionally annotated in the analysis.

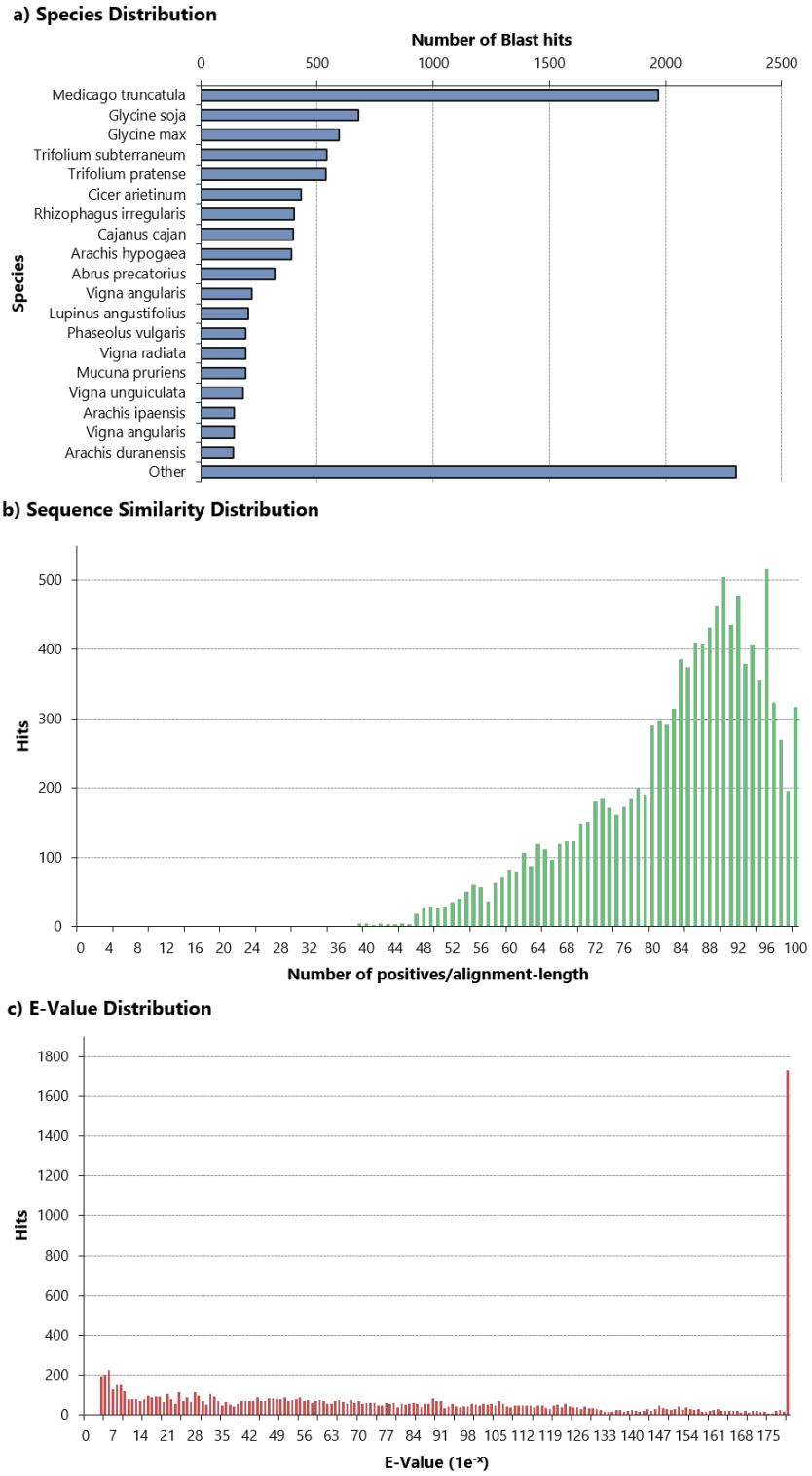


<u>Figure 6.3:</u> Volcano plot of DE transcripts as log Fold Change (logFC) between 0 day and 3 day for VFA treatment RNA samples.

Root tissue data is shown only for *de novo* RPKM sample comparisons. Transcripts are deemed DE if all experimental replicates have an absolute log fold change of 0.585 and false-discovery rate adjusted q-value < 0.05. Graph modified from Degust [183] and made using voom/Limma method [184].

6.2.3 Differentially expressed transcripts have many homologues related to legume model species, particularly *Medicago truncatula*

Once DE transcripts for VFA treatment effects were collated, their transcript isoforms underwent basic local alignment search tool (BLAST) analysis to find genes either in *M. sativa* or closely related genes. The results are shown below for root tissue samples in Figure 6.4. Most DE isoforms were found to have homologues in closely related leguminous species (Figure 6.4a), most particularly *M. truncatula*, and *Glycine* and *Trifolium* species. Numerous successful BLAST hits were found for DE transcripts (Figure 6.4b), and most had extremely low E-values close to zero (Figure 6.4c), indicating these transcripts were more likely to be significant and not to be hits by chance. This was strong evidence that the transcript isoforms were related to BLAST gene hits.



<u>Figure 6.4:</u> BLAST sequencing results for DE transcripts of VFA treatment from *de novo* RNAseq analysis, showing results for root tissue only.

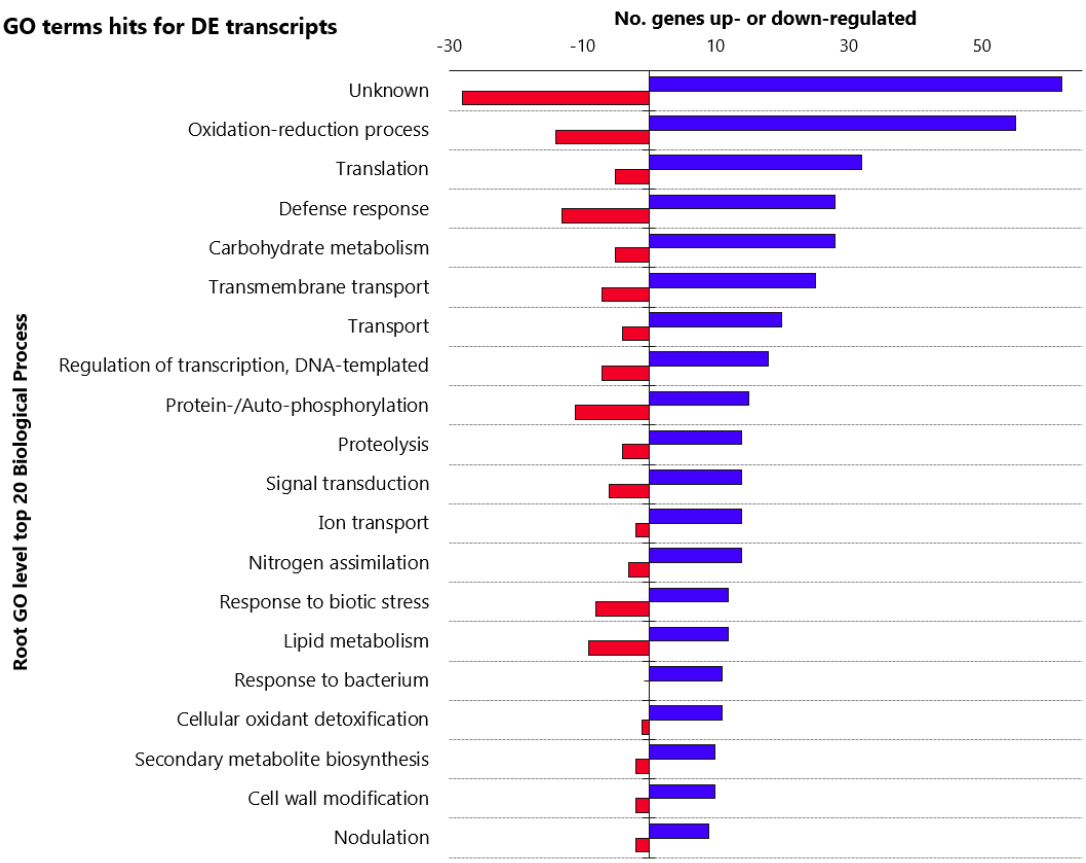
DE transcripts were processed in Blast2GO [185; 186] and using the pipeline transcripts for BLAST result against NCBI's non-redundant NR database [187] with project results as follows; a) Species distribution of number of BLAST hits; b) Sequence Similarity Distribution for hits against alignment length; c) E-Value Distribution of hits.

6.2.4 Biological processes associated with VFA treatment include oxidation-reduction, gene regulation, metabolism, transport and defence

BLAST results of DE transcripts were analysed for their related Gene Ontology (GO) 'Biological Process' terms. Figure 6.5 displays the GO terms for individual DE transcripts from VFA treatment of *M. sativa* roots. The plot shows the number of DE transcripts with their corresponding biological process GO terms that were either upregulated or downregulated. The GO terms demonstrated that the transcriptional changes within the root are wide-ranging. A 3-day treatment of VFA resulted in changes in genes regulating transcription and translation, including those associated with oxidation-reduction. Metabolism and transport were also affected by VFA treatment, with both carbon metabolism and nitrate assimilation changing in respect to the controls. Changes in C:N metabolism were interesting for the increased vegetative yield phenotype detailed in Chapter 5.

Figure 6.5 also indicated changes in responses to defence, stress, and bacteria. This was likely to be a response to symbiotic bacteria such as *Sinorhizobium meliloti* as at this developmental stage nodulation can begin to be established; it is well documented that important nodulation genes and factors are associated with defence responses through their evolution and function [709; 701; 742; 772; 773].

The same GO trends were found within shoot data for metabolism processes, cellular processes and response to stimuli. However, this was at a reduced level due to a lower number of transcripts being DE, (see Figure 6.2).



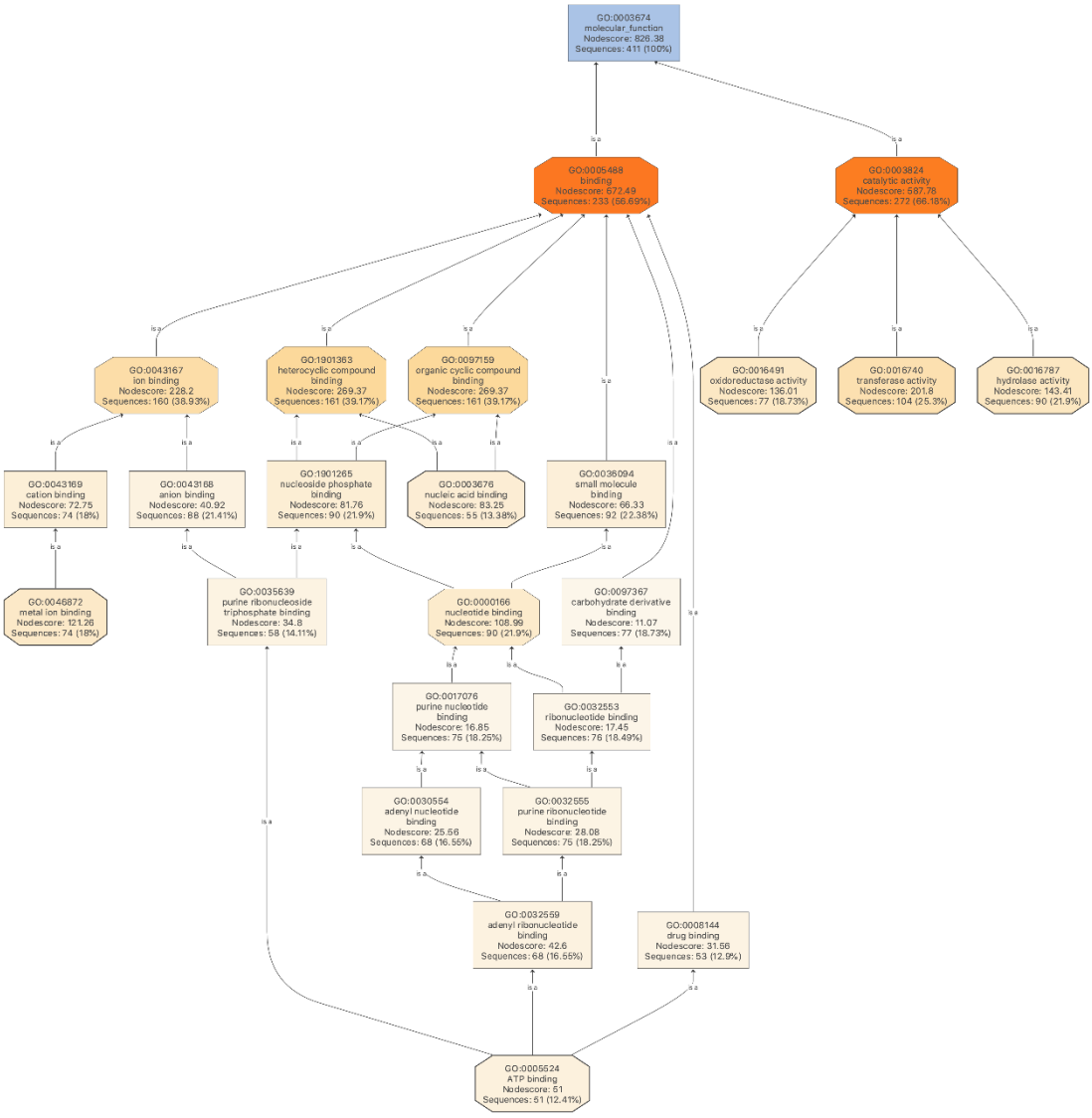
<u>Figure 6.5:</u> 'Biological Process' GO term hits for individual DE transcripts from VFA treatment of *M. sativa* roots.

GO terms were pulled using Blast2GO programme mapping and ran with the EMBL-EBI InterPro library [188], with manual addition from QuickGO [195] and UniProt [196] databases. Bars show genes upregulated (blue) or downregulated (red), indicating the number of DE transcripts with the associated GO term. Only the top 20 GO terms are shown; total GO terms associated with dataset was 142.

6.2.5 Molecular functions associated with VFA treatment include catalytic activity and binding

BLAST results of significantly DE transcripts were also analysed for their related GO 'Molecular Function' terms. Figure 6.6 is a Direct Acyclic Graph (DAG) of molecular function for roots following VFA treatment after 3 days. This indicated huge changes in proteins with catalytic and binding activities, including those associated with transport. This matched the biological process changes of transcriptional and translational regulating genes shown above in Figure 6.5.

As for roots, analysis of shoot tissue DE transcripts found similar molecular function increases, particularly those of hydrolase catalytic activity and binding associated with oxidation-reduction.



<u>Figure 6.6:</u> Direct Acyclic Graphs (DAGs) of GOSlim 'Molecular Function' associated with DE transcripts from VFA treatment of *Medicago sativa* roots.

DAG was made in Blast2Go with Sequence filter of 50, nodescore filter of 1.0, and nodes are coloured by score value.

6.2.6 Metabolism was significantly enriched by VFA treatment, particularly N metabolism

Having collated all significantly DE transcripts, enrichment tests for both 'Biological Process' and 'Molecular Function' were calculated using Fisher's exact test, shown in Tables 6.2 and 6.3 respectively. All enrichment was based on legume gene IDs for the *M. truncatula* reference so could be slightly different when a *M. sativa* reference is available. However, as analysis negated differences found in day 0 VFA and VC, and those in day 0 VC and day 3 VC, it was likely that these calculated fold enrichments were accurate.

From the data in Table 6.2 metabolic and catabolic processes were upregulated in roots, particularly those associated with N metabolism. Reponses to bacteria were also enriched, matching the idea of an effect on nodulation initiation causing such a change. Moreover, cell wall biogenesis and organisation were enriched, which are key factors required for new root development and nodule growth.

In Table 6.3, molecular function enrichment test shows that in the root nutrient transporter activity was highly upregulated with VFA treatment. Moreover, enrichment of serine hydrolase activity was detected, which has wide-ranging catalytic activity in plants [774; 775]. A change in chitin regulatory genes [776; 777] were particularly interesting with DE as follows for each (logFC, q-value); *agglutinin-2* (5.59, 1.30E^{-06}); *BAR-domain-containing protein* (3.22, 1.36E^{-05}); *legume lectin beta domain protein* (5.27, 2.12E^{-05}*); L-type lectin-domain containing receptor kinase IX.1-like* (2.49, 5.82E^{-05}); *chitin elicitor receptor kinase 1-like* (2.48, 9.25E^{-06}); *putative chitinase* (1.49, 2.30E^{-05}).

Many interesting N metabolism related genes were DE as follows for each (logFC, q-value); *ammonium transporter 3 member 1* (2.97, 5.64E^{-05}) and 1-like (5.63, 9.68E^{-06}); *NRT1/PTR 2.6-like* (4.13, 4.76E^{-06}); *NRT1/PTR 4.5* (3.01, 1.32E^{-06}) and *NRT1/PTR 4.5-like* (3.73, 1.59E^{-06}); *Medtr7g098220.1 peptide transporter* (4.09, 3.09E^{-06}); *Nitrite reductase(NAD(P)H) large unit* (3.64, 6.69E^{-07}) and *Nitrite reductase(NAD(P)H) small unit* (3.3, 1.79E^{-04}).

Table 6.2: Fold enrichment of top 20 'Biological Process' for upregulated DE transcripts from VFA treatment of *Medicago sativa* roots.

Enrichment for each category is scored against the *Medicago truncatula* reference and indicated with p-value using Fisher's exact test, using PANTHER analysis [197; 199; 198]. Only DE transcripts with gene ID annotated in mapping were included.

Biological Process	Expected based on reference	Actual fold enrichment	p-value
nitrogen utilization	0.02	87.32	$3.59E^{-04}$
sterol metabolic process	0.46	8.73	$1.30E^{-03}$
cell wall biogenesis	0.25	11.91	$2.30E^{-03}$
organic hydroxy compound metabolic process	0.56	7.17	$2.62E^{-03}$
proteolysis involved in cellular protein catabolic process	1.45	4.14	$3.64E^{-03}$
cellular protein catabolic process	1.46	4.11	$3.78E^{-03}$
steroid biosynthetic process	0.09	22.54	$4.03E^{-03}$
cell wall organization	0.09	21.83	$4.28E^{-03}$
phytosteroid metabolic process	0.11	18.88	$5.60E^{-03}$
lipid homeostasis	0.12	16.63	$7.08E^{-03}$
nitrogen compound metabolic process	0.13	15.19	$8.38E^{-03}$
terpenoid biosynthetic process	0.13	14.86	$8.72E^{-03}$
protein catabolic process	1.77	3.39	$9.35E^{-03}$
macromolecule catabolic process	2.02	2.96	$1.69E^{-03}$
isoprenoid biosynthetic process	0.20	10.12	$1.77E^{-03}$
defence response to bacterium	0.21	9.70	$1.91E^{-03}$
response to bacterium	0.21	9.70	$1.91E^{-03}$
organic hydroxy compound biosynthetic process	0.21	9.57	$1.96E^{-03}$
response to external biotic stimulus	0.21	9.44	$2.01E^{-03}$
response to other organism	0.21	9.44	$2.01E^{-03}$

<u>Table 6.3:</u> Fold enrichment of top 20 'Molecular Function' for upregulated DE transcripts from VFA treatment of *Medicago sativa* roots.

Enrichment for each category is scored against the *Medicago truncatula* reference and indicated with p-value using Fisher's exact test, using PANTHER analysis [197; 199; 198]. Only DE transcripts with gene ID annotated in mapping were included.

Molecular Function	Expected based on reference	Actual fold enrichment	p-value
serine hydrolase activity	0.19	20.85	$5.33E^{-05}$
ammonium transmembrane transporter activity	0.03	69.86	$5.24E^{-04}$
cation transmembrane transporter activity	1.39	5.04	$5.60E^{-04}$
catalytic activity	21.90	1.69	$1.02E^{-03}$
proton transmembrane transporter activity	0.85	5.86	$1.84E^{-03}$
monovalent inorganic cation transmembrane transporter activity	0.85	5.86	$1.84E^{-03}$
ATPase activity, coupled to transmembrane movement of substances	0.53	7.59	$2.14E^{-03}$
solute:proton symporter activity	0.54	7.43	$2.31E^{-03}$
ATPase activity, coupled to movement of substances	0.57	7.02	$2.82E^{-03}$
ion transmembrane transporter activity	1.86	3.76	$2.94E^{-03}$
oxidoreductase activity	4.76	2.52	$3.18E^{-03}$
oligopeptide transmembrane transporter activity	0.31	9.70	$4.03E^{-03}$
peptidase activity, acting on L-amino acid peptides	1.63	3.68	$6.35E^{-03}$
peptide transmembrane transporter activity	0.39	7.65	$7.67E^{-03}$
peptidase activity	1.72	3.49	$8.17E^{-03}$
amide transmembrane transporter activity	0.41	7.33	$8.60E^{-03}$
inorganic cation transmembrane transporter activity	1.32	3.79	$1.11E^{-03}$
Unclassified	109.46	0.89	$1.62E^{-02}$
transporter activity	3.26	2.45	$1.75E^{-02}$
monooxygenase activity	0.98	4.09	$1.77E^{-02}$

6.2.7 Enriched DE transcripts in VFA roots are associated with well-known nodulation regulatory and signalling genes

Many DE transcripts from three days after the VFA treatment were found to be significantly enriched for genes associated with responses to other organisms, including bacteria (see Table 6.2). It was noted that a lot of these have also been categorised as specific early symbiotic root nodule genes in *M. truncatula* in a study by Roux *et al*., (2014) [705]. In this 2014 study they used laser-capture microdissection of roots and nodules coupled with RNAseq to provide a robust list of genes induced early on in nodulation initiation. Table 6.4a, 6.4b, and 6.4c show the significantly DE transcripts from this project which were also defined in their study.

Annotations showed the VFA treatment enriched a vast array of important genes required for the signalling and regulation of nodulation. These included an array of transcription factors (TFs) and domains including *Myb/SANT-like DNA-binding domain protein*, *RING-H2 finger protein ATL52-like*, *AP2-like ethylene-responsive transcription factor*, *WD40 repeat-like protein*, and *zinc finger MYM-type protein 1-like*. Many leucine-rich repeat receptor-like kinases (LRR RLKs) and other receptor kinases were found to be enriched with VFA treatment, for example *LysM domain receptor-like kinase*. Genes required in bacteria and hormone induced plant responses were found, for example *NDR1/HIN1-like protein 10*, *protein RRP6-like 2*, and *cytokinin hydroxylase-like* transcripts. Finally, many nodulation specific genes were enriched such as *nodulation-signaling pathway (NSP) proteins*, *NSP-interacting kinases*, and *nodulins*.

Table 6.4a: Enriched DE transcripts in VFA roots which are putatively classed as highly preferential nodulation regulatory genes and nodule-associated signalling-related genes as in Roux *et al*., (2014) [705].

This includes a description of the protein, available gene/protein IDs, the annotation type, and log fold change (logFC) and q-value for each DE transcript.

Gene/Protein ID	Description	Annotation	logFC	q-value
XP_024635034	*Myb/SANT-like DNA-binding domain protein*	TF MYB	5.35	$1.90E^{-06}$
PNX91228	*putative CC-NBS-LRR resistance protein*	LRR	4.98	$1.23E^{-11}$
ABD33274, AES59362, RHN77255	*RALF-like protein*	Calcium/lipid-binding	4.74	$4.63E^{-06}$
RIA81513	*calnexin*	Calcium/lipid-binding	4.67	$6.36E^{-09}$
RHN49201	*wall-associated receptor kinase-like 20*	RLK	4.60	$3.35E^{-05}$
KEH36571, RHN72042	*CLAVATA3/ESR (CLE)-related protein*	Ser/Thr protein kinase	4.36	$2.44E^{-07}$
KEH28705, RHN58556	*putative LRR-domain, L domain-containing protein*	LRR	4.29	$6.43E^{-05}$
XP_003612592, AES95550, RHN54652	*RING-H2 finger protein ATL52-like*	TF ZnFg C2H2	4.28	1.63^{-75}
XP_024641562	*AP2-like ethylene-responsive transcription factor*	TF AP2/ERF	4.08	1.74^{-04}
XP_003594815, AES65066, RHN73104	*COBRA-like protein 7*	COBRA	4.00	$1.78E^{-07}$
XP_003598348, AES68599, RHN65475	*F-box protein interaction domain protein*	F-box protein	3.75	$4.90E^{-07}$
AES76072, AES76110, RHN52304	*NDR1/HIN1-like protein 10*	NHL	3.67	$2.52E^{-04}$
RHN60433	*disease resistance protein (TIR-NBS-LRR class)*	LRR RLK	3.64	$7.66E^{-06}$
XP_013443270, KEH17295, RHN51739	*cytokinin hydroxylase-like*	CK activated	3.60	$6.29E^{-06}$
XP_013466350, KEH40391, RHN77806	*receptor-like protein kinase*	RLK	3.55	$4.72E^{-06}$
XP_003604023, AES74274	*COBRA-like protein 1*	COBRA	3.53	$7.35E^{-07}$

<u>Table 6.4b:</u> Enriched DE transcripts in VFA roots which are putatively classed as highly preferential nodulation regulatory genes and nodule-associated signalling-related genes as in Roux *et al.*, (2014) [705], continued from Table 6.4a.

Gene/Protein ID	Description	Annotation	logFC	q-value
RGB31681	*calcium-binding protein*	Calcium/lipid-binding	3.49	$1.84E^{-06}$
RHN72504	*probable inactive receptor kinase At2g26730*	RLK	3.42	$1.32E^{-05}$
XP_003613167, AES96125, RHN55010	*L-tryptophan--pyruvate aminotransferase 1*	TAA1-like	3.39	$2.54E^{-04}$
AES69839	*LRR-P-loop containing nucleoside triphosphate hydrolase*	LRR	3.32	$7.54E^{-06}$
AES91737	*F-box/kelch-repeat protein*	F-box protein	3.32	$6.92E^{-06}$
XP_024637477	*disease resistance protein (TIR-NBS-LRR class)*	LRR	3.24	$1.65E^{-05}$
EXX59026	*WD40 repeat-like protein*	TF WD	3.22	$1.01E^{-04}$
XP_024631685, RHN72543	*mitogen-activated protein kinase kinase kinase 18-like*	STY	3.14	$2.55E^{-05}$
ABD28520	*protein RRP6-like 2*	CK activated	3.09	$9.33E^{-05}$
XP_013451548, KEH25576, RHN50766	*ankyrin repeat/protein kinase domain-containing protein 1*	TF ERF	3.06	$2.97E^{-05}$
AES95938	*disease resistance protein (TIR-NBS-LRR class), putative*	LRR RLK	3.01	$1.91E^{-05}$
RZB96753	*probable LRR receptor-like Ser/Thr-protein kinase*	LRR RLK	2.99	$5.78E^{-05}$
KHN26259	*zinc finger MYM-type protein 1-like*	TF Zn finger	2.95	$1.31E^{-04}$
XP_013451184, KEH25223, RHN50327	*protein NSP-interacting kinase 1*	NSP	2.94	$3.50E^{-05}$
RHN42361	*kinase RLK-Pelle-WAK-LRK10L-1 family*	RLK	2.89	$1.29E^{-04}$
RIA84146	*Ca2+:H+ antiporter*	Calcium/lipid-binding	2.87	$8.02E^{-05}$
AES60803	*F-box plant-like protein*	F-box protein	2.78	$1.71E^{-04}$
XP_013457946, KEH31977, RHN63702	*putative LRR-containing protein*	LRR RLK	2.78	$1.60E^{-04}$
RIA97789	*ARM repeat-containing protein*	E3 ligase	2.72	$2.11E^{-04}$
XP_013445632	*G-type lectin S-receptor-like Ser/Thr-protein kinase*	Ser/Thr protein kinase	2.71	$1.84E^{-04}$
AES73438	*Plant regulator RWP-RK*	NLP	2.70	$1.87E^{-04}$

Table 6.4c: Enriched DE transcripts in VFA roots which are putatively classed as highly preferential nodulation regulatory genes and nodule-associated signalling-related genes as in Roux *et al*., (2014) [705], continued from Table 6.4b.

Gene/Protein ID	Description	Annotation	logFC	q-value
KEH38435	*Rpp4C3*	CK activated	2.69	$1.22E^{-04}$
RIA81779	*YIF1-domain-containing protein*	TF AP2/ERF	2.69	$1.26E^{-04}$
AES61923, RHN81250	*C3HC4-type RING zinc finger protein*	TF Zn finger	2.68	$1.90E^{-04}$
XP_024633471.1	*LysM domain receptor-like kinase 3*	LysM receptor kinase	2.68	$1.41E^{-04}$
XP_024625794	*putative receptor-like protein kinase*	RLK	2.66	$4.17E^{-05}$
RHN81081	*proline-rich protein 1-like*	PRP	2.63	$5.03E^{-05}$
PF04909	*nodulin-6*	NIP	2.56	$2.00E^{-04}$
XP_003615114, AES98072, RHN56135	*nodulin-26*	NIP	2.56	$6.80E^{-05}$
XP_013450575, RHN49450	*L-type lectin-domain containing receptor kinase IX.1-like*	RLK	2.49	$5.82E^{-05}$
XP_013462891, KEH36925, RHN72571	*chitin elicitor receptor kinase 1-like*	LysM receptor kinase	2.48	$9.25E^{-06}$
XP_003601076.1, AES71327	*nodulation-signaling pathway 2 protein*	NSP	2.11	$1.43E^{-05}$
XP_024641514, AES76606, RHN52721	*putative NF-X1-type zinc finger protein NFXL1-like protein*	NFX1-type zinc finger	1.75	$1.68E^{-05}$
XP_013460228, KEH34259, RHN67624	*non-specific phospholipase*	Phospholipase A2	1.52	$4.71E^{-06}$
XP_024625319	*U-box domain-containing protein 33 isoform X1*	MtPUB	1.37	$1.14E^{-04}$
XP_003631134, AET05610, RHN43936	*probable inactive receptor kinase At1g48480*	Kinase	1.15	$1.75E^{-04}$
XP_003616008, AES98966, RHN56723	*CBL-interacting serine/threonine-protein kinase 11*	Calcium binding, Ser/Thr protein kinase	1.04	$1.40E^{-04}$
RHN48771	*NDR1/HIN1-like protein 1*	NHL	0.88	$3.71E^{-05}$

6.2.8 qRT-PCR confirmed a subset of DE transcripts which are associated with nodulation

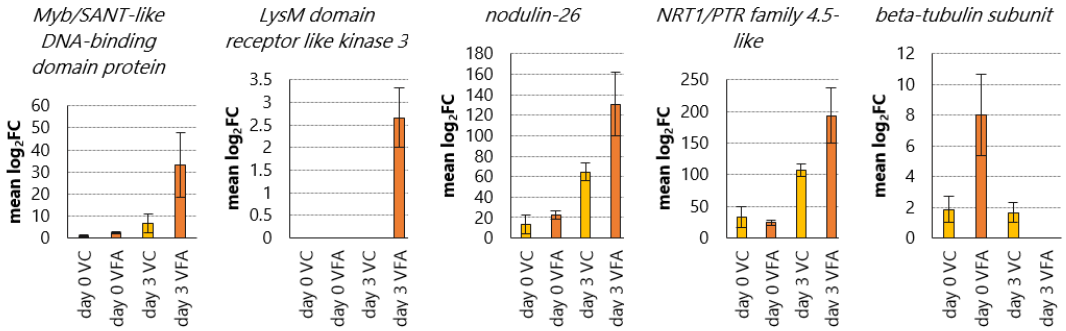
A subset of root DE transcripts were confirmed for their expression using qRT-PCR; similar expression patterns between RNA-seq data and qRT-PCR analysis would validate the previous association of VFA with nodulation effects. Three important genes for nodulation-associated signalling and regulation were chosen, *Myb/SANT-like DNA-binding domain protein*, *LysM domain receptor like kinase 3*, *nodulin-26*, as well as the N metabolism gene *NRT1-PTR family 4.5-like*. The *beta-tubulin* gene was also measured using qRT-PCR as a gene with reduced expression after 3 days of VFA treatment. Figure 6.7 shows a table of transcript ID tested, with RNAseq and qRT-PCR fold change values (Figure 6.7a). Below this, each gene is plotted individually for RNA-seq data (Figure 6.7b), and qRT-PCR analysis (Figure 6.7c).

All genes showed similar expression patterns between different treatments and timepoints. For example, for *Myb/SANT-like DNA binding domain protein* gene in both treatments at day 0 was low in expression, then for day 3 VC increased in transcript number. However, for day 3 VFA transcript level were many folds higher than the other treatment timepoints. Each of these genes were validated for their RNA-seq expression values using qRT-PCR with *ACTIN2* as the reference housekeeping gene.

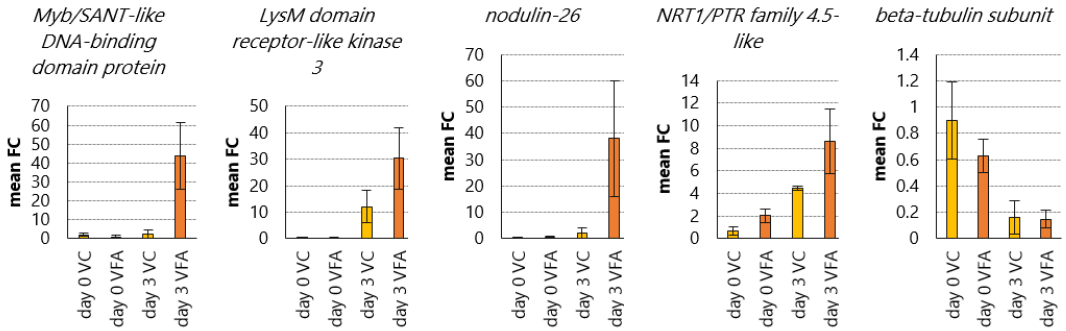
a) Transcript ID and expression data

Transcript ID	Annotation	RNAseq mean log_2FC				qRT-PCR mean FC			
		Day 0		Day 3		Day 0		Day 3	
		VC	VFA	VC	VFA	VC	VFA	VC	VFA
TRINITY_DN157351_c1_g1	Myb/SANT-like DNA-binding domain	2.02	0.94	2.18	43.86	0.96	2.39	6.68	33.08
TRINITY_DN415738_c0_g1	LysM domain receptor-like kinase 3	0	0	0	2.67	0.33	0	11.98	30.37
TRINITY_DN164623_c1_g1	nodulin-26	13.37	22.62	64.69	130.75	0.33	0.43	2.12	38.09
TRINITY_DN170549_c0_g1	protein NRT1/ PTR FAMILY 4.5-like	33.76	24.33	107.50	193.20	0.68	2.05	4.41	8.59
TRINITY_DN154692_c3_g1	beta-tubulin subunit	1.86	8.00	1.67	0	0.90	0.63	0.16	0.15

b) RNA-seq expression level



c) qRT-PCR expression level



<u>Figure 6.7:</u> Quantitative reverse transcription PCR (qRT-PCR) of a subset of differentially expressed (DE) transcripts to confirm RNA-seq in *Medicago sativa*

a) Table of ID and Annotation for five DE transcripts obtained from RNA-seq for *Medicago sativa* root tissue with treatments of either fulvic acid (VFA, orange) or the control (VC, yellow). RNA-seq was carried out on whole root RNA samples taken on day of treatment (day 0) or three days after treatment (day 3). Mean expression level of three independent experimental samples is provided for both RNA-seq and qRT-PCR. b) Bar charts showing mean log_2 fold change in expression (log_2FC) of the five DE transcripts. c) Bar charts showing mean fold change in expression (FC) relative to the reference gene *ACTIN2* of the five DE transcripts. In all cases error bars represent standard error of the mean.

6.3 DISCUSSION

6.3.1 *De novo* transcriptome assembly of *Medicago sativa* was successful

The above analysis is based on the successful *de novo* transcriptome assembly of *M. sativa*, shown in Table 6.1. This assembly mirrors other recent studies carried out in the forage crop [722; 723; 725; 724; 726-730] assessing transcriptional changes in relation to other treatments including stress responses. Such studies can provide information to improve the *Medicago sativa* gene index 1.2 [360], which can progress the genetic resources available for future studies in this important global forage crop.

There was a large overlap in the genes detected in *de novo* analysis which were also detected when mapped to A17 [702; 707] or R108 [178] reference accessions directly. As *M. sativa* is not yet fully sequenced and annotated it is likely the tables of BLAST results and GO terms, found in Figures 6.4, 6.5 and 6.6 and Tables 6.2 and 6.3, are not exhaustive. Some DE transcripts without a BLAST *M. sativa* or legume homologue match are therefore missing from this analysis. These DE transcripts may be of particular interest to study in subsequent work to characterise new genes.

6.3.2 Fulvic acid treatment causes increased transcription for metabolic processes with upregulated transporter activity predominantly in the root tissue

This study shows that FA as VFA can induce substantial transcriptional change in *M. sativa* after only three days. Significant transcriptional changes occurred in both shoot and root tissues, see Figure 6.2. Metabolism and responses to biotic and abiotic stimuli were up-regulated in both tissues after VFA treatment, as discussed in 6.2.4. Molecular functions which were affected following VFA treatment included catalytic and binding activities, particularly for transport and oxidation-reduction

processes, as discussed in 6.2.5. The GO analysis provides evidence that VFA very quickly effects crucial pathways in both C and N metabolism, as well as cell wall modification. This rapid transcriptional effect helps to induce the later yield effect in vegetative tissue found in Chapter 5.

The root had higher amounts of DE transcripts than shoots, see Figure 6.2 and Appendix D Figure D2. This could be caused by the method of application, with VFA and VC being applied to the soil. The higher number of DE transcripts may be due to the roots being the site of more transcriptional change as it is the site of VFA uptake, thus it is affected more promptly and excessively. In addition, the enrichment of N metabolism is more likely to be shown in the root as N uptake is in the roots as nitrate and ammonium [72] then transported up to the shoots as the main tissue for assimilation in both higher and lower order plants [778]. Analysis of soil in Chapters 3 have shown soil to be low in nitrate level for no inhibition of nodulation to occur which is ~ 16 mM for *M. truncatula [779]* and above 10 mM for *L. japonicus* [780]; soil in this study was ~ 8.8 mM in conventional soil water analysis, and ~ 5.53 – 9.02 mM with ion-selective sensor analysis. Changes in N metabolism in roots of legumes is closely associated with increases in nodulation-signalling during initiation of symbiosis [781]. The quick response in transcription in the roots provides evidence for why there is a larger biomass increase after VFA treatment through stimulated N supply to the legume via nodules or uptake by the roots.

6.3.3 Fulvic acid treatment causes enriched upregulation of nodulation regulatory and nodule-associated signalling-related genes

The above transcription enrichment tests shown in Table 6.2 and 6.3, plus the nodulation experiments in Chapter 5, provides strong evidence that VFA can readily induce nodulation when compared to controls. Genes in the root which are significantly induced following VFA treatment overlap with those in studies of early initiation of nodulation in other legumes [700; 709; 703; 719; 720; 716; 706; 714]. Many DE transcripts in Tables 6.4a,

6.4b, and 6.4c have been characterised for their role in nodulation as follows; LysM-type receptor-like kinases are perceive early *Rhizobium* signals [741; 749-752; 747; 782; 783; 754]; many leucine-rich repeat receptor-like kinases including *CLAVATA* protein homologues signal root development and nodulation induction [784-789]; AP2/ERF transcription factors controls nodule number and differentiation [790-793]; nodulin is crucial in early nodule development [794-799; 765; 800; 801; 714; 802; 803] including in *M. sativa* [764; 804-806]. Important chitin regulatory genes are also detected to be changed in their expression by VFA treatment, discussed in 6.2.6. This may affect lipochitooligosaccharide recognition as the key signal in initiating legume-*Rhizobium* symbiosis [807-811]. Other defence-related genes such as *Pathogenesis-related proteins* are increased which can be induced in early symbiotic infection, before adequate *Rhizobium* suppression, rather than being in relation to a pathogen response [709; 701; 742; 753; 782; 735; 773].

The increase in transcription of these genes upon VFA treatment is a strong indication that it is associated with inducing early nodulation in *M. sativa*. This could be by influencing the plant itself in its response to symbiosis, for example a priming effect of VFA for subsequently inducing infection by the symbiont [812-814]. This would match the effect shown in *Pisum sativum* for soil primed with HS increasing nodulation and mycorrhizal colonisation in roots [646]. Fulvic acid may be able to change the C:N metabolic balance of the plant and thus impact on the regulatory mechanisms of promoting symbiotic nodulation processes [815]. Or the effect could be a consequence of the treatment on the symbiont causing a nodule number increase. VFA may contain a specific nutritional aid, not adequately controlled for in VC application, which boasts symbiotic *Sinorhizobium* growth in soil and thus makes nodulation happen more rapidly [816; 817]. Or similarly, VFA may decrease the inhibitory role of N in soil on nodulation and thus also encourage nodulation to occur with symbiont and plant [818; 779]. This is unlikely due to the low N content of the soil used in testing, but should be considered.

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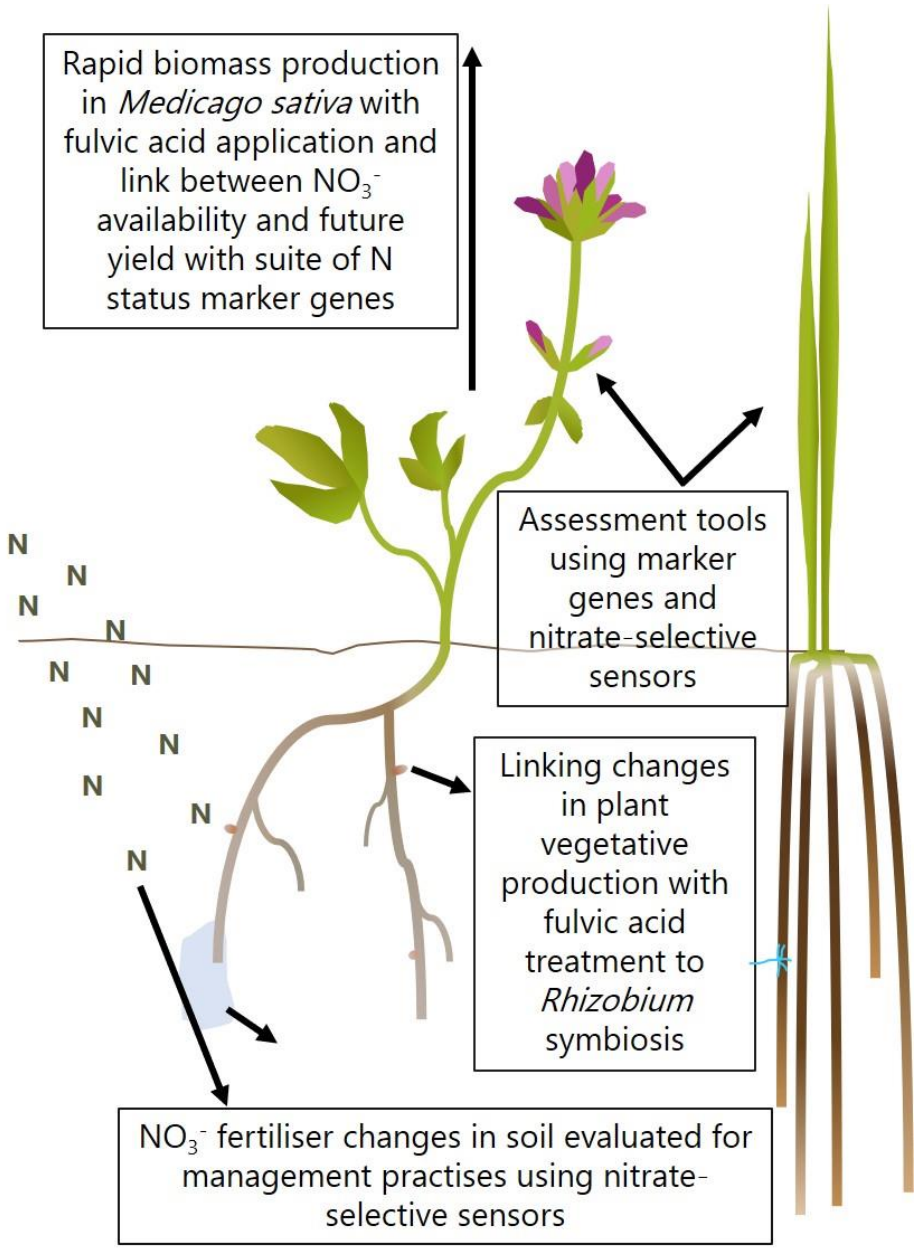
Chapter 7: General Discussion

7.1 SUMMARY OF RESULTS WITH IMPLICATIONS

The overall goal of this project was to study connections between N input and final vegetative biomass in a range of forage crops, particularly *Lolium perenne*, a *Festulolium* hybrid, and *Medicago sativa*. Strategies were developed to test this relationship with the aim of increasing biomass with lowered inputs in future forage crop cultivation. In addition, the project succeeded in providing underpinning evidence to help high-intensity, temporary forage growers, such as BAGCD members, plan their crop management regarding N fertiliser use. The results compared to targets for improved forage crops in Figure 1.4 are shown in Figure 7.1. Below is a discussion of the research in the context of the project area hypotheses, along with the final points for discussion on each of the main project areas. Herein, I will cover N status marker genes as assessment tools from Chapter 3; Management practices influencing soil N profiles using NO_3^--selective sensors from Chapter 4; Fulvic acid as a biostimulant in forages from Chapters 5 and 6.



<u>Figure 7.1:</u> Results compared to targets for improved forage crops in Figure 1.4.

Adapted from Capstaff and Miller (2018) [18], showing the main results of the thesis compared to targets for improvement in forage crop research.

7.1.1 N status marker genes have use across forage crop industry

Hypothesis for Chapter 3: Expression of N status genes is reliably related to crop production and soil N status.

Through the study of marker genes in *Lolium*, *Festulolium*, and *Medicago*, the results in Chapter 3 provide a greater understanding of how the NO_3^- supply in the environment can affect NUE associated gene expression in forage crops. The different patterns of N status marker genes in relation to NO_3^- concentration show that evidence from N metabolism from model plant studies can be applied to less studied crops like forages. This was especially true for the genes *NIR* and *NADH-GOGAT* from *Arabidopsis* studies [377; 378; 134; 133], showing parallel expression in the grasses (see Figures 3.1, 3.4, and 3.5). Being able to relate this information in the field for grasses for optimal plant growth and metabolism to soil N levels is valuable for the high intensity agriculture performed on such crops. In particular, gene expression analysis can help to understand sense and response of NO_3^- in low or high N supply environments in a range of grasses, in temporary and permanent grassland. Although gene expression testing was not successful in *M. sativa*, the development of confirmed efficiency gene primers is still of use to studies in this legume. For example, the gene primers could be used to assess *M. sativa* response to pathogens known to affect C or N metabolism in legumes [819-822].

High variation of soil N in temperate fields such as those in the UK [391-397], was accounted for by testing of plant tissue. With current soil testing it is conventional to test only 3 – 5 soil cores per field each year randomly across a site. In this study three samples per field were also taken, but within each sample there are around 25 – 50 individual grass leaf blades. Through gene expression analysis of only three marker genes, it is possible to gain a picture of the crop's N status and representative soil NO_3^- value across many fields in only a few days. This study also found a general trend where the higher the predicted soil NO_3^- from the gene expression analysis then the higher the subsequent later yield of the field in t ha^{-1} (see Figure 3.10), although this does not correlate as strongly as actual extractable

soil NO_3^-. This is likely due to yield being affected by more factors, especially that of biotic and abiotic stress differences across years unaccounted in the model. More sampling across more years may provide improved data for using marker genes to accurately predict yield as well as actual extractable soil NO_3^-.

Grass tissue sampling is quick and easy, as long as samples can be flash frozen and stored adequately before processing. Soil samples can be difficult to standardise, with compaction making such samples difficult to access. Moreover, soil cores are often split into different depth layers as N levels vary from shallow to deep soils. By using leaf tissue, which is known to have networks of systemic signals from root tissue that penetrate these depths, the analysis reflects the integrated true available N to the plant, not just what can be detected in soil cores. The development of handheld PCR kits in the field [416-419], should make such a routine procedure on farms more possible. This development of a suite of NUE status marker genes could also easily be applied to breeding programmes, which are already equipped with the techniques required to analyse such tissue effectively in a short timescale. Furthermore, they could be used alongside soil column experiments discussed in Chapter 4 to further relate plant growth to changes in soil profiles.

It should be noted that there are some limitations with soil columns. It can be difficult to mirror field soil conditions with variables including changes in soil layers and compaction levels. The differences in soil layers from sand to loam to clay can alter water movement, and compaction in UK farms affects N fertiliser applications. To improve this system, any future experiments could try to replicate these extra factors in soil columns, especially by trying to mirror the conditions to match the soil conditions of BAGCD member farms. Although the soil columns in this project do not match such factors, the profile data is superior to other methods such as hydroponics and gel-based media; these methods cannot reproduce such data as water movement is unconstrained. Repeating such experiments with soil layers and compaction included would be more applicable to forage crop cultivation, as could more depths of samples being included for analysis. Moreover, using gene expression analysis on crops grown in

columns to see if these match with the field data could provide more evidence of how well soil columns relate to the situation within the field.

7.1.2 Studying the field soil N availability provides more detail for growers to pursue precision agriculture techniques

Hypothesis for Chapter 4: Using nitrate-selective sensors in soil columns with grass and lucerne will provide valuable data on plant-soil interactions of management practices.

Soil column experiments using NO_3^--selective sensors were successful in tracking NO_3^- through soil profiles following different management practices. This provided valuable data for real-time soil changes following NO_3^- application, defoliation of vegetative tissue, and the role intercropping may play in plant-soil interactions for these practices. The use of NO_3^--selective sensors could help in the advancement of precision agriculture by providing detailed information of fields [823; 514; 517], where other conventional methods are too labour-intensive, expensive, and provide only one time measurements. Furthermore, this study using soil columns suggest there is little leaching of NO_3^- when forage crops are present with the current management practices. This means that despite the high NO_3^- application, little NO_3^- is actually lost from the base of the column when plants are grown, suggesting that leaching in temporary systems such as BAGCD maintained sites may not be as problematic as previously proposed. This is likely to only be in cases where crops are able to efficiently take up the applied NO_3^-, as is possible in well-maintained glasshouse conditions.

The most striking observation in these measurements was the detection of a NO_3^- 'burst' in the middle soil column region following defoliation of *L. perenne*. Possible reasons for the NO_3^- 'burst' could include decreased root uptake through changes in transpiration rate [538-542], reduced plant N uptake [209], or changes in N composition to other non-NO_3^- forms [212; 531; 530]. However, these seem unlikely as the concentrations of NO_3^- are exceedingly high, reaching around 60 – 75 mM. Such dramatic changes

not found in other levels of the soil profile, and well-conserved across experiments, suggests this is a potential grass defoliation phenomena previously unobserved. It is unlikely that the NO_3^- 'burst' would be a problem for growers, as its detection drops overtime suggesting uptake (or reuptake) of the NO_3^-, with little evidence of leaching to the lower level through either NO_3^--selective sensor data or conventional testing. Furthermore, the lack of a detected 'burst' when intercropped with *M. sativa* also provides evidence that this is grass specific, with the legume's own root system or potential to change soil microbial populations being possible reasons for no detection.

It is feasible that C-containing compounds exuded by the root could interfere with the NO_3^--selective sensor measurements. However, malate has been tested and shown not to affect readings [518], although, it is possible that there may be other organic anions that interferes with sensor readings. Nevertheless, this would not explain why any potentially interfering anions are not detected in the intercropping system. It seems reasonable to suspect that the lack of detection of the 'burst' is due to root architecture differences, already of note in erosion studies of intercropping grasses and legumes and also found in permanent pastures [88-92]. As the lack of NO_3^- 'burst' detection in intercropping experiments may be due to improved diversity in root architecture of *M. sativa* with *L. perenne*, compared to monocropping of *L. perenne* alone, studies should be carried out with more grasses with cultivars known to have better root systems. For example, measuring for NO_3^- 'burst' across grass species especially *Lolium* compared to both *Festuca* and *Festulolium* hybrids may help to elucidate this as they have different root architectures from breeding programmes. Moreover, if the lack of detection burst in intercropping is due to microbial population changes from legume crop, then testing other forage legumes including *Trifolium* and *Lotus* may provide evidence of this, as discussed in Chapter 4.

7.1.3 New management practices including fulvic acid application could be advantageous in forage crops

Hypothesis for Chapter 5: Application of the commercially available biostimulant fulvic acid (FA) improves forage crop production.

&

Hypothesis for Chapter 6: RNA-seq will provide evidence of early differentially regulated genes in either shoot or root tissue upon fulvic acid application.

An increase in vegetative biomass in *Medicago sativa* was observed upon treatment with FA across laboratory and field trials. Through experimental analysis in Chapter 5 and RNA-seq analysis in Chapter 6, it is clear FA also has an impact on soil microorganisms such as *Sinorhizobium* species, either directly or indirectly. The lack of biomass increases in grasses (see Figures 5.1 and 5.2) suggests FAs role of N-fixing bacteria may be at least partially responsible for the increase of biomass in legumes. It seems very likely that FA may replicate C-containing exudates usually released by plants to aid in symbiosis initiation, which in turn stimulates growth and activity of this bacteria. This leads to increased nodulation signalling which makes nodulation symbiosis more likely, with the end result of higher nodule number which directly increases vegetative growth. There was no increase in protein or chlorophyll concentration suggesting that the enhanced vegetative growth is not due to protein storage but due to increased growth rate. This can be achieved by increase N causes more leaf expansion and, therefore, more C capture via photosynthesis may occur to ensure a C:N balance, which seems to be the case in both laboratory and field conditions. This could be tested through the use of NUE status marker genes linked to C metabolism such as *RBCS*, *GLN*, and *NADH-GOGAT* from Chapter 3. This would mean that although the suite of genes was not conducive for assessing soil N status of *Medicago* the markers may have use as a proxy for C:N metabolism rate.

Although this study provides strong, indirect evidence for the FA effect on the nodule and bacteria, the above hypotheses for increased vegetative growth through nodulation symbiosis encouragement requires testing.

More direct methods could be used particularly in testing legume nodulation mutants, or with inoculation with *Rhizobium* nodulation mutants [824-827]. If when nodulation is not possible, especially at an early initiation stage, FA does not incur a later vegetative phenotype in the plant then a mode of action can be more formally hypothesised. Reporter bacteria and measurements of N fixation such as through acetylene reduction assays will make such ideas testable.

It was thought for many decades that humic substances, like FA, have a crucial active ingredient or 'hormone', such as an auxin-like molecule [653; 654; 607; 643; 655]. However, based on the analysis in this project, no such molecule was detected as both commercial applications were found to be remarkably different from one another. It is possible that many studies have exaggerated results due to a lack of standardised nutritional controls especially for FA elemental contents, and although solutions such as FA do promote growth responses it is likely to be a mixture of many compounds which provide this increased yield. It would be interesting to screen FA in relation to the growth, or inhibition, for a range of bacterial strains, as this may provide more evidence of which classes of compounds are most necessary for the effect. To assess whether FA is a considerable new management practices in legume farming, large-scale and standardised testing of a range of globally sourced FAs is required. By performing an extensive screen of FAs it may be possible to find common nutritional components in solutions, which is not possible in a study of only two compounds. Controls such as those used in this study could then be introduced to further pinpoint particularly important fractions, which may lead to targeted synthesis of the molecules found in such portions for use in later studies.

7.2 OPEN QUESTIONS

Although this research has provided both specific outcomes and general information of relevance to forage growers in the UK, there are still many open questions which could be further investigated.

7.2.1 Improving tools for tackling nitrogen use efficiency need to be accessible to growers

The strategies adopted in this study to assess plants and soil, primarily the suite of N status genes and use of NO_3^--selective sensors, provide tools that can be used in research for many applications. More research into grass regrowth post-defoliation in regard to N metabolism using gene expression could be deployed alongside more thorough analysis of soil NO_3^- profile changes. Additionally, these tools could be applied to other crops, especially cereals, which are in the same family as forage grasses, or leafy vegetables, both of which are important in the forage industry. Cereals are complicated by the fact that the crop tissue of interest for NUE (defined in Table 1.2), is grain and not whole vegetative biomass, therefore more analysis may be required before deployment in these crops. Leafy vegetables are similar to forage crops as crop tissue of interest is whole vegetative biomass, and so may be more readily applicable with these tools.

The suite of genes tested and confirmed for field use have a range of applications, particularly in prompt assessment of new hybrid cultivars for their metabolic capacity under many parameters, especially for abiotic stresses, such as drought and severe temperature fluctuations.

NO_3^--selective sensors look set to provide a whole range of exciting prospects for future studies, especially due to recent hopes that they can be made using solid-state technology, like some new types of commercial pH sensors. This development, along with improvements in data analysis and sharing, will greatly improve data acquisition in soil column experiments, but will also aid in establishing field-based measurements to compare to those shown in this project.

7.2.2 Management practices require thorough testing to demonstrate their effectiveness as grassland research is not standardised

It is clear from this project that new management practice could be implemented in UK forage farming, such as the addition of FA to legume cultivation. FA application could be provided as a seed coat treatment, or mixed in with inoculum already applied to legume seeds. Such practices require large-scale testing in industry, where standardisation of yield measurements can be achieved across multiple studies around the world over many seasons.

In addition, the management practice of intercropping may be a highly effective way to negate leaching in soils, however due to problems with harvest intervals and competition, it is still in the relatively early stages of practice for modern intensive agriculture. Moreover, the soil column data in this study suggests little evidence of NO_3^- leaching when forage crops are present, as either a monocrop or as an intercrop, with increased biomass of *L. perenne* when *M. sativa* is present. As animal feeds can be a mix of grasses and legumes, forage crop cultivation is therefore an ideal system to test more how leaching is affected by rooting systems.

A key problem with this is the range of forage crop cultivation methods found in the UK and Europe, discussed in Chapter 1. Soil columns are one way to relatively quickly assess management practices whilst changing parameters. Yet, a crucial problem will still remain in how to apply results to forage croplands and grasslands. It may be necessary for studies on leaching of N fertiliser to be carried out in both temporary and permanent field systems, for data to be truly meaningful in each circumstance. This is important when using research to assign policy to agriculture – it is clear a one-fits-all solution to forage cultivation in regard to fertiliser application is unlikely. Nevertheless, the improvements described in this project should contribute to more rapid analysis of forage crops in multiple cultivation systems. Below are the main findings for this thesis work, followed by the recommended future testing based on them.

Main findings:

- Festulolium testing during growth using suite of N status marker genes was able to accurately assess both soil NO_3^- levels and future yield, instead of conventional testing.
- No detected soil changes of nitrate level were found for intercropping experiments following defoliation, with intercropping of forage grass with legumes, such as *M. sativa*, with decreased N fertiliser application gaining similar biomass.
- An FA application showed improved yield in legumes, particularly in *M. sativa*, and was linked to nodulation affect.

Recommended future testing:

- Testing of more grass species using N status marker genes can prove their use in fields, especially with more field trials included for applicability to growers and breeders.
- Further analysis for NO_3^- leaching in field can be carried out with NO_3^--selective sensors, as unobserved as a problem in soil columns with current management practices.
- Applying FA to legume forage crops, such as with testing in seed inoculum as applied by seed suppliers currently, can be further tested for investigating the shown increased vegetative growth.

7.2.3 Fertiliser use must be curtailed ahead of resource depletion

As forage crop cultivation methods in the dried forage industry of the UK are one example of the huge levels of N fertiliser application in agriculture, it is clear the sector requires vast changes promptly. N fertiliser use is energy and resource exhaustive, so research in agricultural practices with high N use with the aim to decrease N use are vital to study, so N usage is reduced substantially and quickly. N fertiliser resource depletion due to tot-al energy resource strains are on the horizon, so such research should be prioritised to curtail use in the UK. Through studying a resource demanding systems such as forage crops in high intensity, temporary

grasslands, it may be possible to apply use efficiency advances in other crop systems. The results of this project have shown how yields can be measured for forage crops in relation to their N application, with each providing a potential way to decrease fertiliser applications. This is particularly true for the rise in precision agriculture, with the tools of N status marker genes and NO_3^--selective sensor soil data improving when and where N fertiliser is applied in the future.

7.3 CONCLUDING REMARKS

Temperate forage crops are exceptionally important crops, and when grown for dried forage production as high-intensity, temporary cropland they can have substantial fertiliser applications. As an increase in efficiency is required for this agricultural practice, the aim of this thesis was to investigate three strategies to increase yields and assessments in UK forage crops.

Through validating a suite of N status marker genes for use in forage crops, field testing has provided more evidence of how crop production is related to soil N levels. This has provided data directly applicable to grower and breeder needs, and in addition, provided knowledge for N metabolism studies in grasses.

Nitrate-selective sensors have provided extensive data of soil N profile changes following different management practices. This data has been shown to be superior to conventional testing. Additionally, the detection of a NO_3^- 'burst' following defoliation of grasses localised to one area of soil columns requires further investigation.

Lastly, the implementation of a new management practice of fulvic acid application has shown to be very useful in increasing vegetative biomass in lucerne. This yield increase was shown in many growth conditions, including the field. As nodulation was also affected, in physiological experiments and transcriptome analysis, there is scope to investigate fulvic acid's role in both plant and microbial growth.

This project has provided practical information to aid forage crop growers in farming more efficiently, especially with providing tools to assess both plant and soil conditions. This work provides new avenues of work into management practices of forages which are underrepresented in current research.

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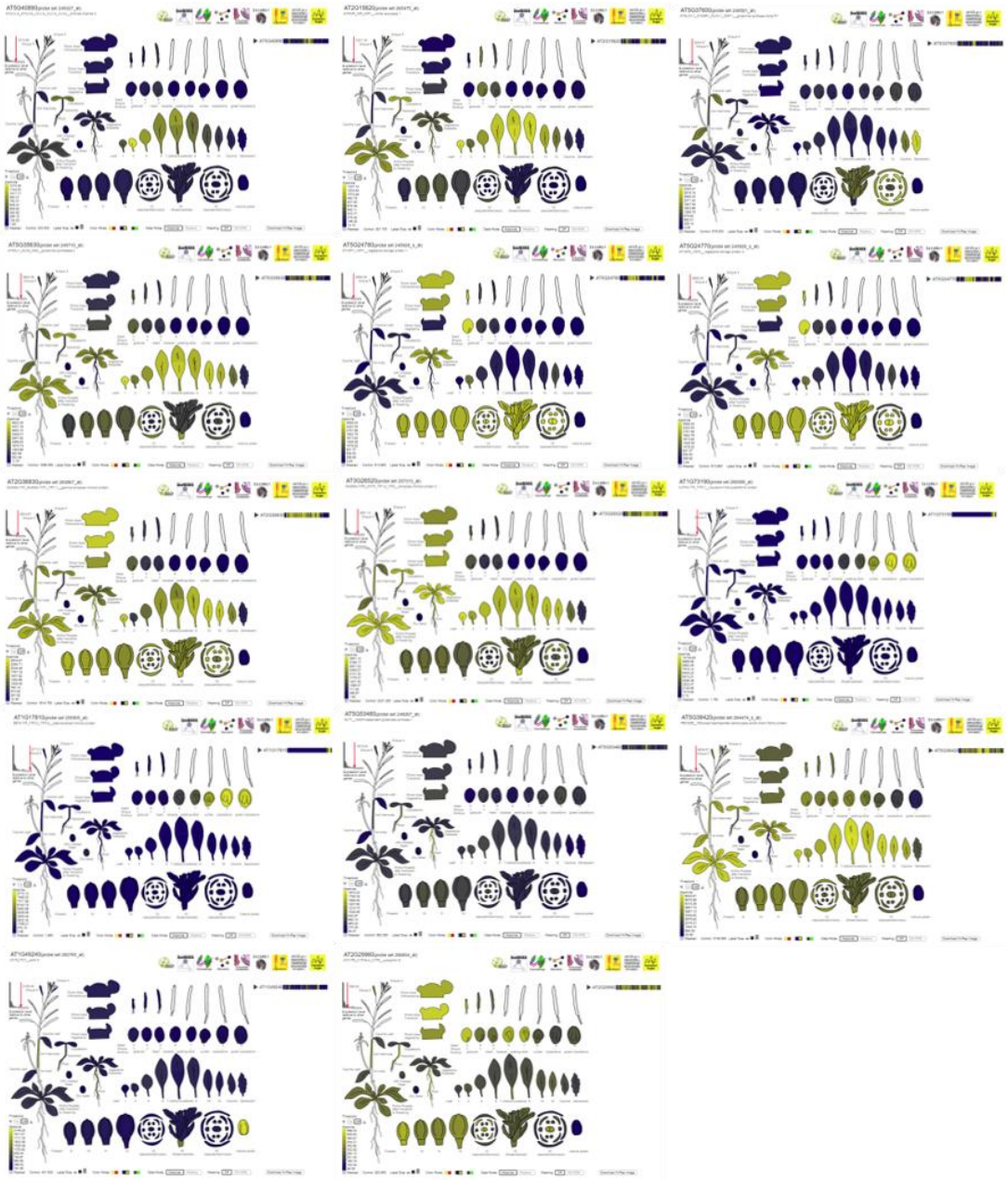
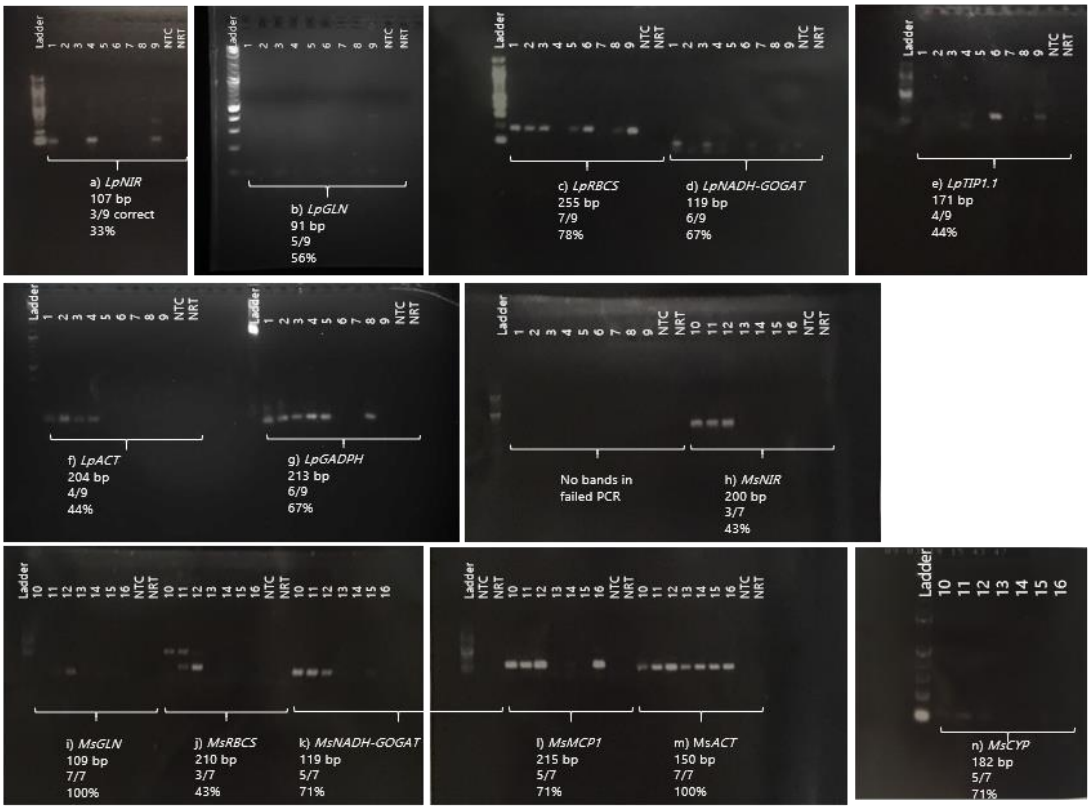
Appendix A: Chapter 3 supplemental figures



<u>Figure A1:</u> Arabidopsis eFP Browser [128-130] images for expression candidate N status genes.

Genes were as follows; *CLCa* (AT5G40890); *NIR* (AT2G15620); *GLN1* (AT5G37600); *GLN2* (AT5G35630); *VSP1* (AT5G24780); *VSP2* (AT5G24770); *TIP1.1* (AT2G36830); *TIP1.2* (AT3G26520); *TIP3.1* (AT1G73190); *TIP3.2* (AT1G17810); *NADH-GOGAT* (AT5G53460); *RBCS2B* (AT5G38420); reference genes *ACTIN8* (AT1G49240) and *CYP5* (AT2G29960).



KEY
1 = *L. perenne*
2 = *L. multiflorum*
3 = *F. arundinacea*
4 = *F. pratensis*
5 = *L. multiflorum x F. arundinacea*
6 = *L. mulitflorum x F. pratensis*
7 = *L. perenne x F. pratensis*
8 = *A. aristatum*
9 = *P. pratense*
10 = *M. sativa* cv. Daisy
11 = *M. sativa* cv. Luzelle
12 = *M. sativa* cv. Gea
13 = *L. corniculatus*
14 = *O. sativa*
15 = *T. pratense*
16 = *T. repens*

<u>Figure A2:</u> Forage crop RT-PCR screen of developed N status marker gene primers electrophoresis gel photos.

PCR conducted as in Section 2.1.11 with numbering as indicated in key.

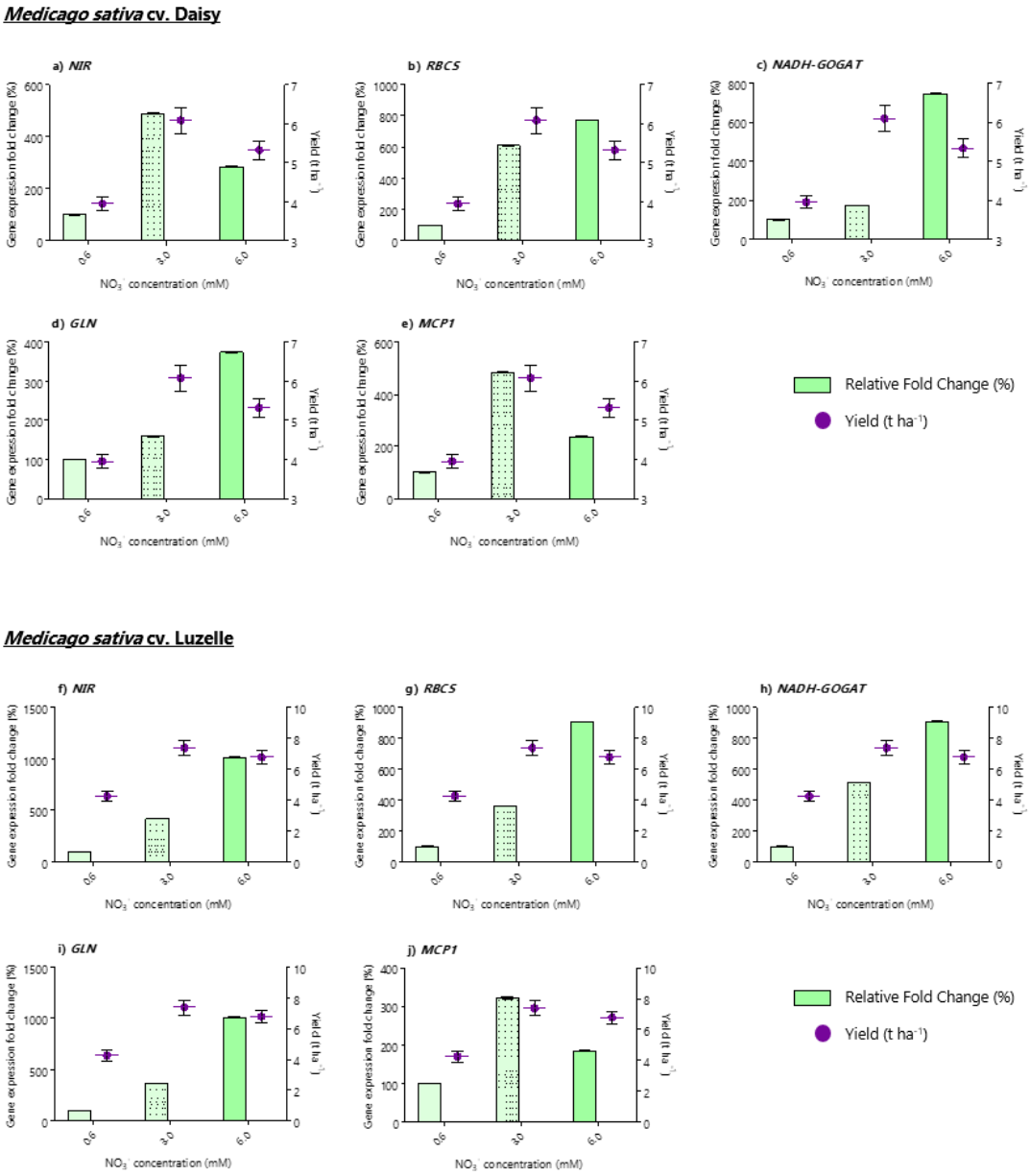


Figure A3: Gene expression patterns of N status marker genes in *Medicago sativa*, cv. Daisy and Luzelle, vegetative tissue at three weeks. Expression was calculated as the geometric expression percentage of independent $CaNO_3$ and KNO_3 experiments relative to lowest NO_3^- concentration (0.6 mM shown as 100 % in pale green). Bars are denoted for increasing concentration using darkening shades of green, and error bars are standard deviation of expression using two reference genes with 10 biological replicates, in two independent experiments. Expression of genes showed to vary dramatically in relation to NO_3^- concentration with agreement between cultivars. Genes tested in Daisy and Luzelle were; a) and f) is *NIR*; b) and g) is *RBCS*; c) and h) is *NADH-GOGAT*; d) and i) is *GLN*; e) and j) is *MCP1* (*Medicago truncatula TIP1* homologue).

<u>Table A1:</u> R Script outlined for Gaussian Process (GP) fitting model of N status marker gene expression.

Script uses both 2018 and 2019 glasshouse data detailed in Section 2.1.20.

```
#Packages installed and opened, theme set to 'theme_bw'
install.packages("installr")
library(installr)
install.packages("tidyverse")
install.packages("cowplot")
install.packages("ggplot2")
install.packages("data.table")
install.packages("GauPro")
library(tidyverse)
library(cowplot)
library(ggplot2)
library(data.table)
library(GauPro)

theme_set(theme_bw())

#Functions assigned for normalising gene expression
geoMean <- function(x) {
  return(exp(mean(log(x))))
}

load_data <- function(file.name) {# read data and formatting
  df <- fread(file.name)
  names(df)[names(df)=='2.delta.ct'] <-  'two.delta.ct'
  df$gene <- factor(df$gene, levels=c('NADH', 'NIR', 'TIP'))
  df$hk.gene <- factor(df$hk.gene, levels=c('ACT', 'GADPH'))
  df$cv <- factor(df$cv, levels=c('hykor'))

  df[, mean.delta.ct:=geoMean(two.delta.ct), by=.(environment, cv,
NO3, treatment, gene, rep)]
  df$hk.gene <- NULL
  df$two.delta.ct <- NULL
  df <- unique(df)
  df$sample_id <- paste(df$environment, df$cv, df$treatment,
'NO3=', df$NO3, df$rep, sep='-')

  return(df)
}

my.norm <- function(x) {
  y <- x / mean(x)
  return(y)
}

standardize <- function(v) {
  v <- (v - min(v)) / (max(v)-min(v))
}

unstandardize <- function(w, vmax, vmin) {
  v <- w*(vmax-vmin)+vmin
}

opt.rescale <- function(curr.opt.df, r) {
  curr.opt.df$expt.val <- curr.opt.df$mean.delta.ct.norm * r
```

```
  curr.opt.df$dnorm <- dnorm(curr.opt.df$expt.val,
mean=curr.opt.df$yhat, sd=curr.opt.df$se, log=T)
  ll <- sum(curr.opt.df$dnorm)
}

#Importing data and assign above factors
getwd
setwd('/RMG2018_2019')
df <- load_data('RAWdataboth.csv')
df$year <- as.factor(df$year)
df.h <- df[df$cv=='hykor', ]
df.h[, mean.delta.ct.norm:=my.norm(mean.delta.ct),
by=.(environment, cv, treatment, year, gene)]

#Plot of 'mean.delta.ct.norm' versus 'NO3'
ggplot(df.h, aes(x=NO3, y=mean.delta.ct.norm, color=year))+
  geom_point(alpha=0.5, size=2)+
  facet_wrap(~environment+gene, scales='free_y', ncol=3)
rm(df)

#Functions assigned for building model with glasshouse data using
'yhat' and 'xpred', including min and max
p.list <- list()
gp.list <- list()
pred.list <- list()
df.g <- df.h[df.h$environment=='greenhouse']

df.g$standard.NO3 <- standardize(df.g$NO3)
xmax <- max(df.g$NO3)
xmin <- min(df.g$NO3)

for (curr.gene in unique(df.g$gene)) {
  print(curr.gene)
  curr.df <- df.g[df.g$environment=='greenhouse' &
df.g$gene==curr.gene]
  x <- matrix(curr.df$standard.NO3)
  y <- matrix(curr.df$mean.delta.ct.norm)

  ymax <- max(y)
  ymin <- min(y)
  y=standardize(y)

  gp <- GauPro(X=x,Z=y, verbose=2)

  xpred <- seq(min(df.g$standard.NO3), max(df.g$standard.NO3),
length.out=50)
  yhat <- gp$pred(xpred)
  UL <- gp$pred(xpred) + 2* gp$pred(xpred,T)$se
  LL <- gp$pred(xpred) - 2* gp$pred(xpred,T)$se

  out.df <- data.frame('gene'=curr.gene, 'NO3'=x,
'mean.delta.ct.norm'=y)
  out.df.pred <- data.frame('gene'=curr.gene, 'xpred'=xpred,
'yhat'=yhat, 'UL'=UL, 'LL'=LL)

  out.df$NO3 <- unstandardize(out.df$NO3, xmax, xmin)
  out.df$mean.delta.ct.norm <-
unstandardize(out.df$mean.delta.ct.norm, ymax, ymin)

  out.df.pred$xpred <- unstandardize(out.df.pred$xpred, xmax,
xmin)
```

```
  out.df.pred$yhat <- unstandardize(out.df.pred$yhat, ymax, ymin)
  out.df.pred$UL <- unstandardize(out.df.pred$UL, ymax, ymin)
  out.df.pred$LL <- unstandardize(out.df.pred$LL, ymax, ymin)


#Plot of GP fitting models of each gene with 'yhat' and 'xpred'
  p <- ggplot(out.df.pred, aes(x=xpred, y=yhat))+
    geom_line()+
    geom_ribbon(aes(ymin=LL, ymax=UL), alpha=0.3)+
    geom_point(data=out.df, aes(x=NO3, y=mean.delta.ct.norm))+
    ggtitle(curr.gene)

  p.list[[curr.gene]] <- p
  gp.list[[curr.gene]] <- gp
  pred.list[[curr.gene]] <- out.df.pred
}
plot_grid(plotlist=p.list)

#Save GP fitting models for use in field data
saveRDS(gp.list, file='./ok_greenhouse_modelsboth.rds')
saveRDS(pred.list, file='./ok_greenhouse_models_preddfboth.rds')

#Load the GP fitting and run with field data
pred.list <- readRDS('./ok_greenhouse_models_preddfboth.rds')
pred.df <- do.call('rbind', pred.list)
pred.df$se <- (pred.df$UL - pred.df$yhat) / 2

df.f <- df.h[df.h$environment=='field']
field.points <- split(df.f, by=c('NO3', 'rep'))

out.df.list <- list()
PlotList <- list()
curr.no3 <- unique(pred.df$xpred)[1]
for (i in 1:length(field.points)) {
  curr.df <- field.points[[i]]
  measured.NO3 <- curr.df$NO3[1]
  sample.label <- paste0(curr.df$NO3[1], '_', curr.df$year[1],
'_', curr.df$rep[1])
  print(sample.label)
  curr.no3 <- unique(pred.df$xpred)[1]

  curr.df.list <- list()
  j=1
  for (curr.no3 in unique(pred.df$xpred)) {
    curr.pred.df <- pred.df[pred.df$xpred==curr.no3, ]
    curr.opt.df <- merge(curr.pred.df[, c('gene', 'yhat', 'se')],
curr.df[, c('gene', 'mean.delta.ct.norm')], by='gene')

    best.LL <- list()
    best.LL[['objective']] <- opt.rescale(curr.opt.df=curr.opt.df,
r=1)

    x = seq(min(pred.df$xpred), max(pred.df$xpred), length.out=50)
    prior = dnorm(curr.no3, mean=measured.NO3, sd=10, log=T)
    best.LL[['objective']] <- best.LL[['objective']] + prior

    out.df <- data.frame('label'=sample.label,
'expt.NO3'=measured.NO3, 'assessed.NO3'=curr.no3,
'LML'=best.LL[['objective']])
    curr.df.list[[j]] <- out.df
    j <- j+1
```

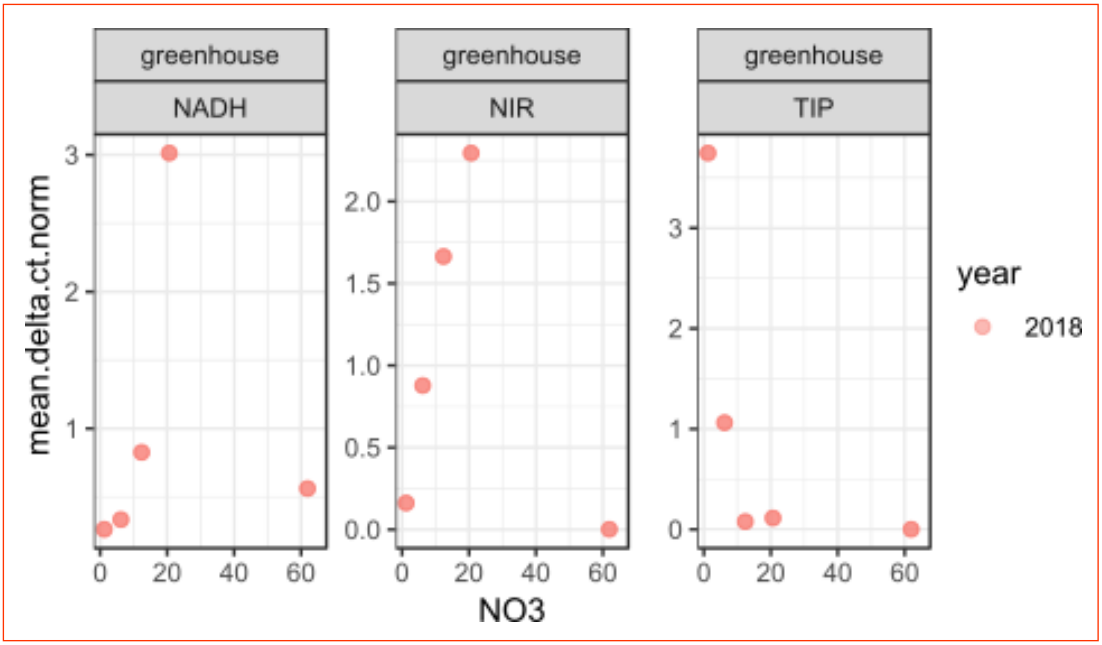
```
  }
  out.df <- do.call('rbind', curr.df.list)
  out.df.list[[i]] <- out.df

  curr.df$best.assessed <-
out.df$assessed.NO3[out.df$LML==max(out.df$LML)]
}
out.df <- data.table(do.call('rbind', out.df.list))
out.df <- unique(out.df)

out.df$ML <- exp(out.df$LML)
out.df[, evidence:=sum(ML), by=.(label)]
out.df$ML <- out.df$ML / out.df$evidence

#Plotting of field values for individual genes using GP fitting
model using 'yhat' and 'xpred'
p.grid <- plot_grid(plotlist = PlotList)
p.grid

#Plotting of field values for combined genes using GP fitting
model using 'P(D|NO3)' for relative values of 'yhat' for each
gene, and 'predicted equivalent greenhouse NO3'
test.df <- out.df[out.df$label=='22.24_2018_1',]
ggplot(out.df, aes(x=assessed.NO3, y=ML))+
  geom_bar(stat='identity')+
  facet_wrap(~label)+
  xlab('predicted equivalent greenhouse NO3')+
  ylab('P(D | NO3)')
```



<u>Figure A4:</u> Normalised expression data 'mean.delta.ct.norm' of 2018 glasshouse grown *Festulolium*.

NADH-GOGAT ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') normalised expression data was calculated from glasshouse measurements in relation to two reference genes, to generate values for 'mean.delta.ct.norm' against known soil 'NO3'. Details found in Sections 2.1.12 and 2.1.13.

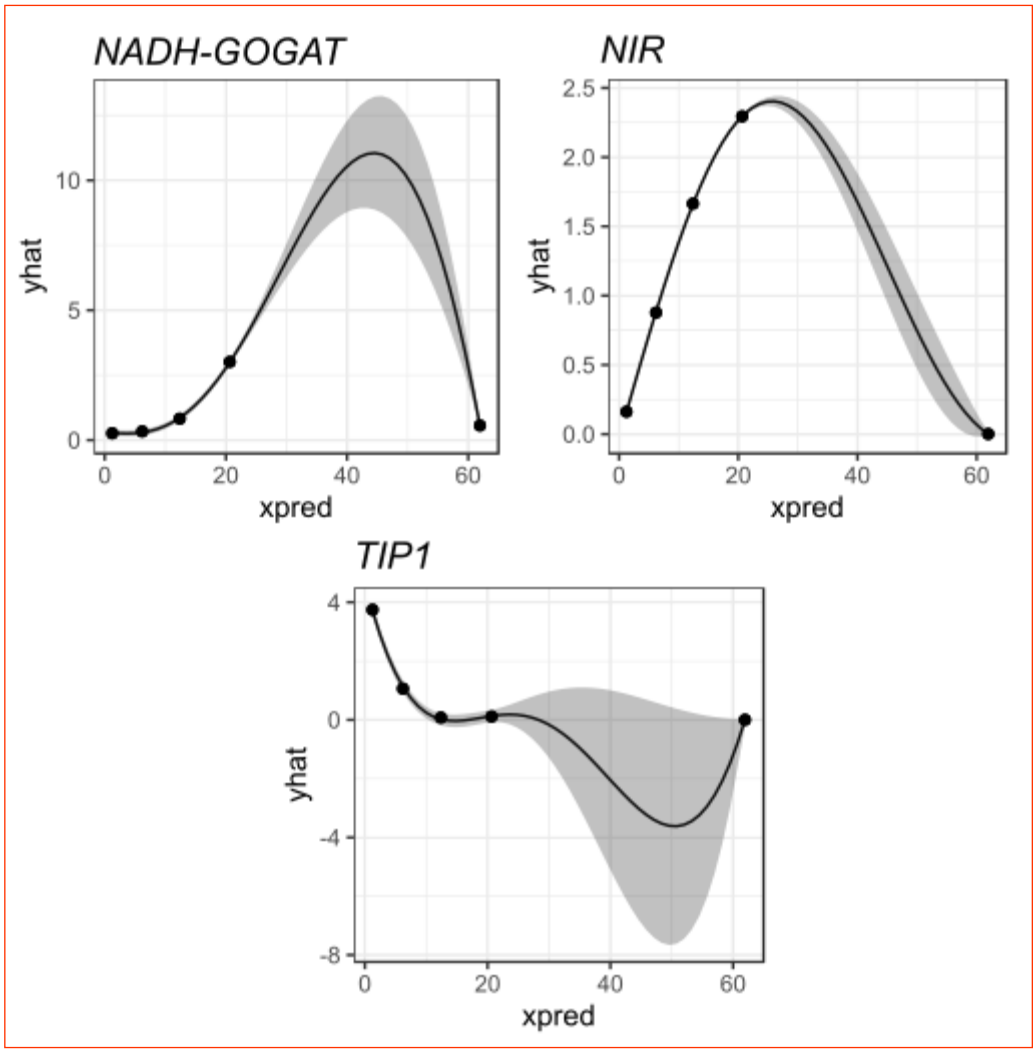
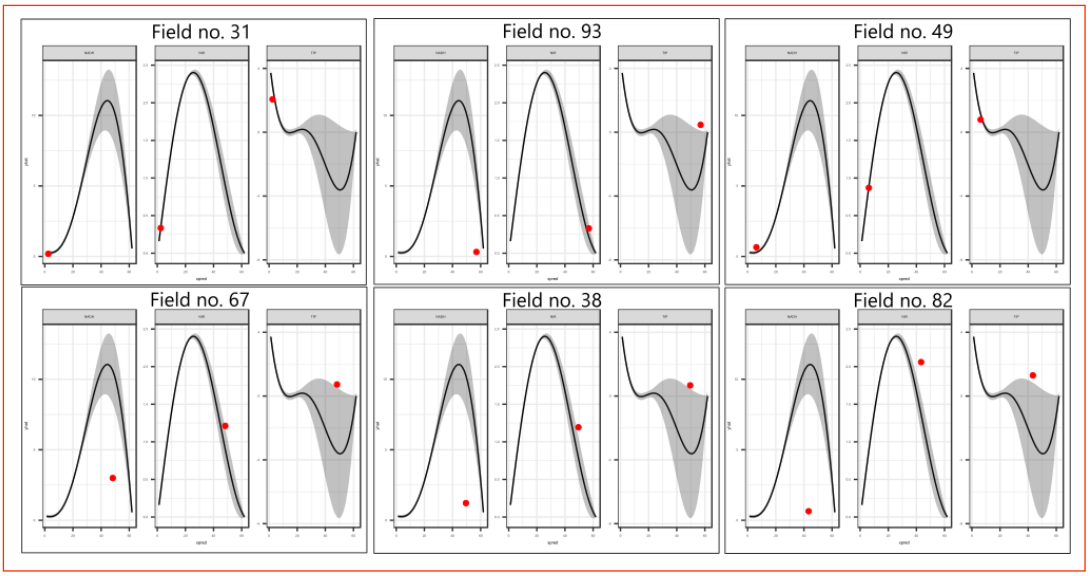
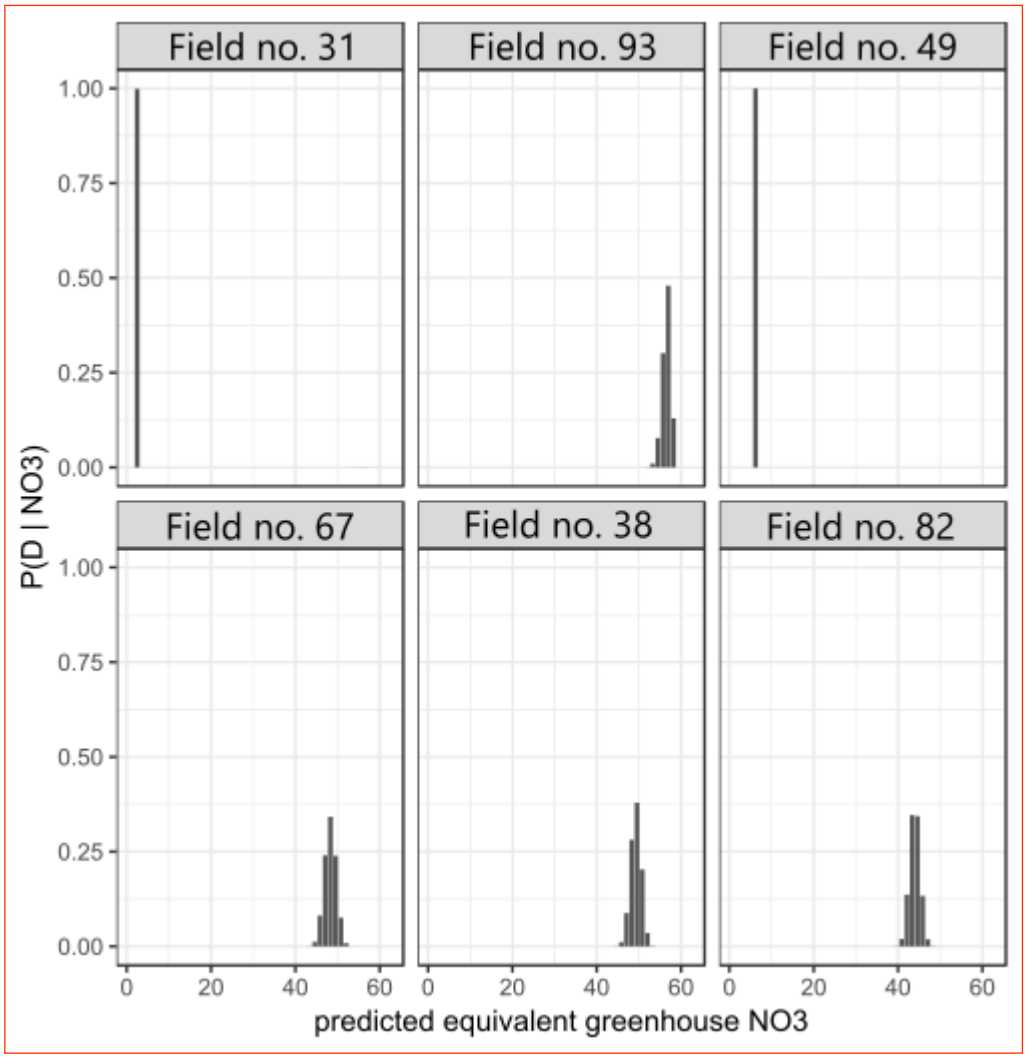


Figure A5: Gaussian model of 2018 glasshouse grown Festulolium. *NADH-GOGAT* ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') normalised expression data generated from glasshouse data with a Gaussian model applied to individual genes; y-axis is 'yhat' is the mean of all gene expression values for 'mean.delta.ct.norm' as in above figure, with minimum and maximum value provided; this is required for *NADH-GOGAT* and *NIR* to ensure the model can distinguish the low NO_3^- and high NO_3^- despite the 'mean.delta.ct.norm' being similar in range. The x-axis is termed 'xpred' and is the predicted soil NO_3^- from actual values provided; when field data is analysed with the model it is the 'xpred' which will be deduced from the raw geometric expression data provided. Error region in light grey ribbon shows the upper and lower limit of the model, with the small the region indicating the smaller the range of error between predicted soil NO_3^- results. Details found in Section 2.1.20.



<u>Figure A6:</u> Analysis of individual gene models for 2018 field grown *Festulolium*.

Field sample expression data from 2018 was calculated in relation to two reference genes and the normalised gene expression for each gene calculated using a glasshouse tissue generated Gaussian model expression, found in Figure A5; 'yhat' is the mean values for 'mean.delta.ct.norm', and x-axis 'xpred' is predicted soil NO_3^-. Details found in Section 2.1.20.



<u>Figure A7:</u> Analysis of combined gene models for 2018 field grown *Festulolium*.

Field sample combined normalised gene expression analysed with glasshouse 2018 generated Gaussian model. Here y-axis is 'P(D|NO3)' which is a symbolic derivative for simple expressions of individual gene 'xpred' values shown in Figure 3.8, thus indicating a single area of expression to relate to x. The x-axis is again 'xpred', here termed 'predicted equivalent greenhouse NO3'. This provides a visual representation of predicted concentrations in the field soil with the higher the 'P(D|NO3)' bar points the more reliable the interpretation of predicted soil NO_3^-.

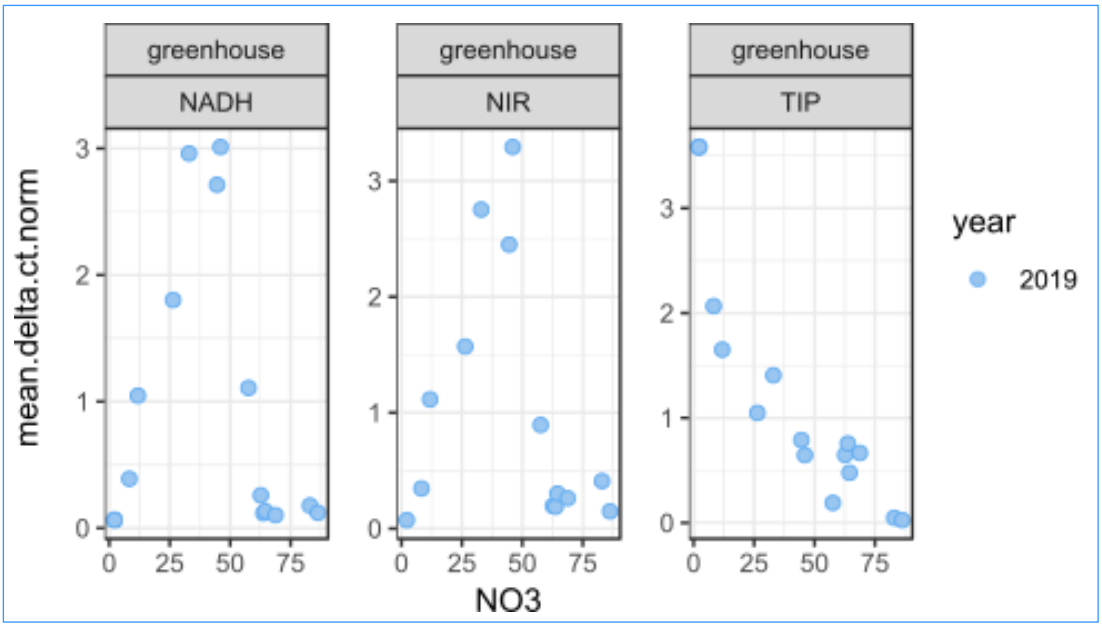
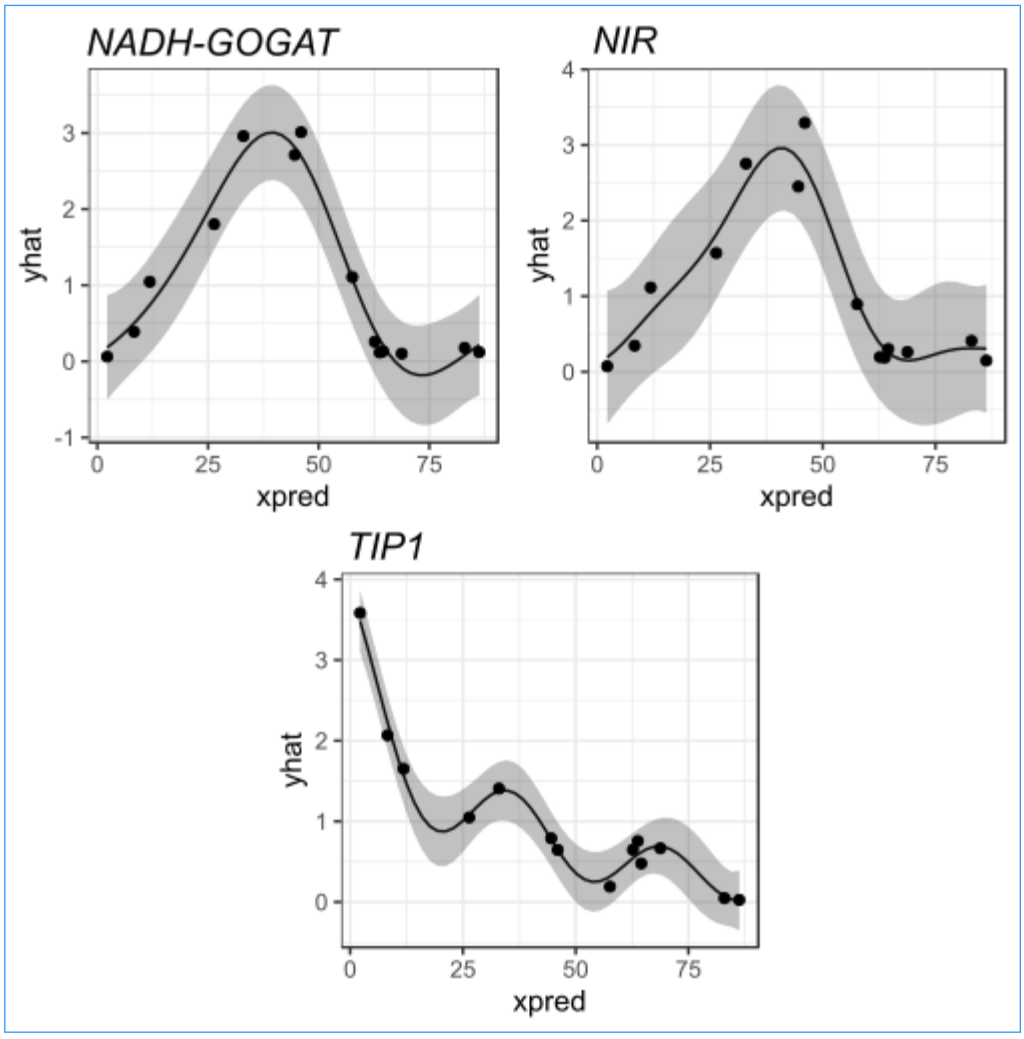
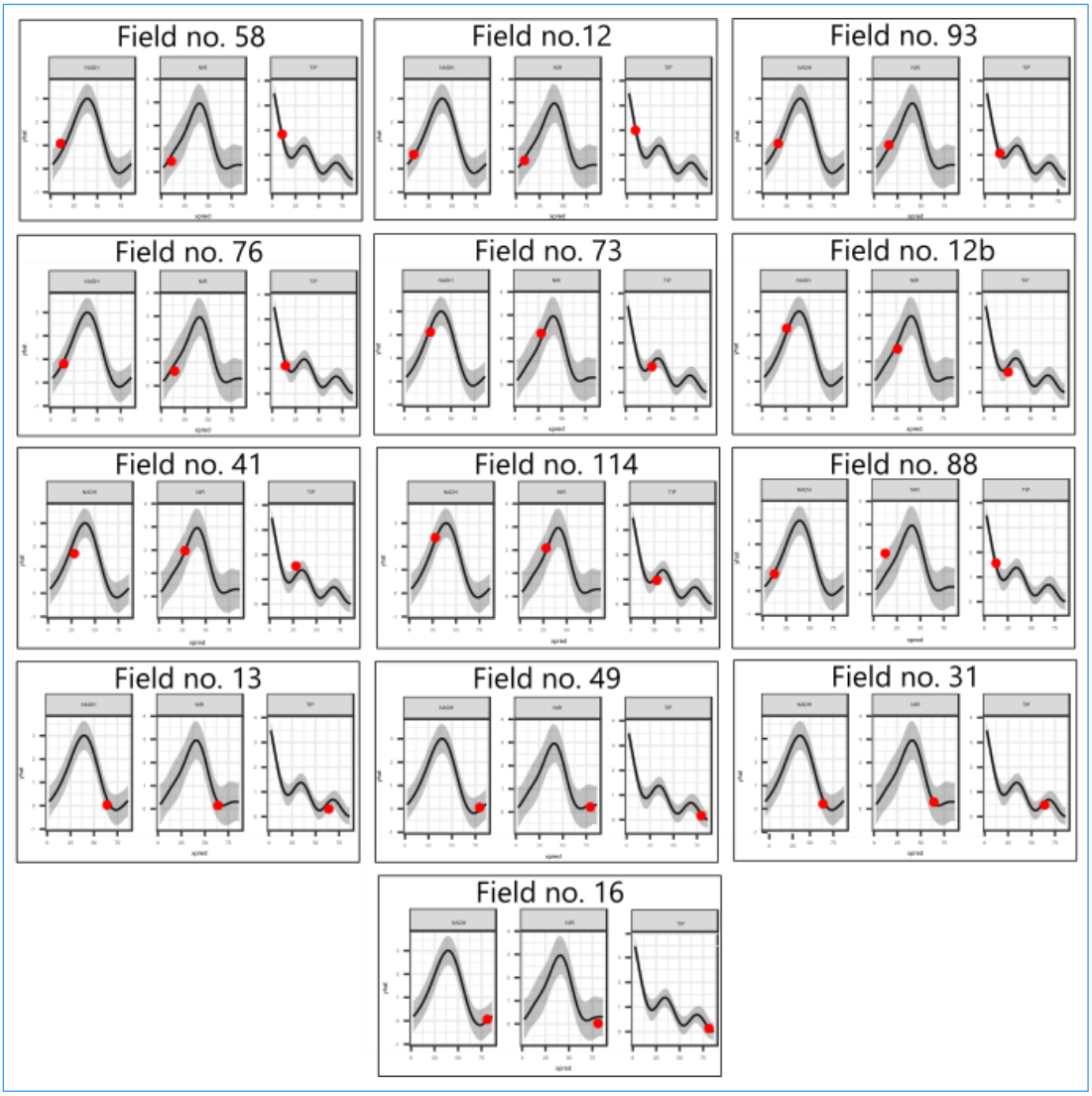


Figure A8: Normalised expression data 'mean.delta.ct.norm' of 2019 glasshouse grown *Festulolium*.

NADH-GOGAT ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') normalised expression data was calculated from glasshouse measurements in relation to two reference genes, to generate values for 'mean.delta.ct.norm' against known soil 'NO3'. Details found in Sections 2.1.12 and 2.1.13.

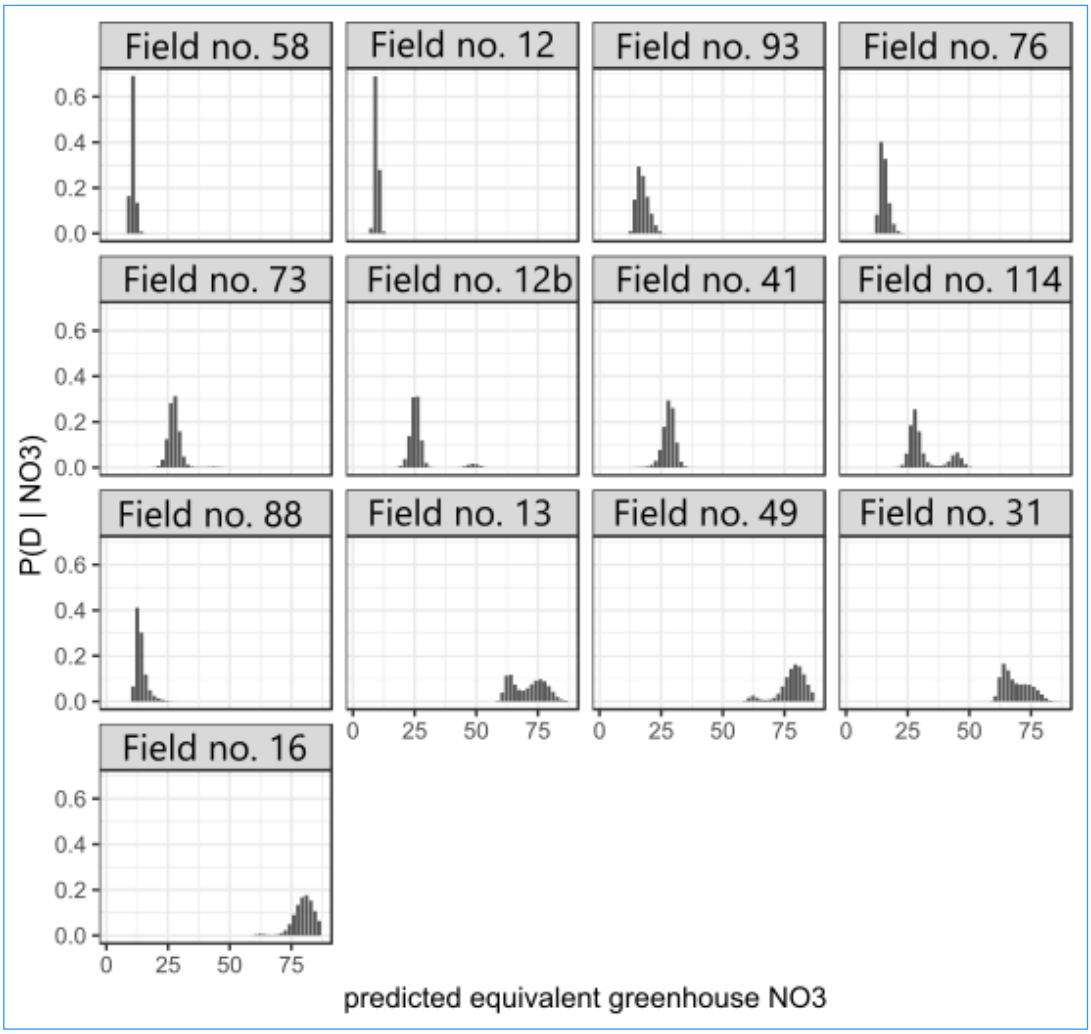


<u>Figure A9:</u> Gaussian model of 2019 glasshouse grown Festulolium. *NADH-GOGAT* ('NADH'), *NIR* ('NIR') and *TIP1* ('TIP') normalised expression data generated from glasshouse data with a Gaussian model applied to individual genes; y-axis is 'yhat' is the mean of all gene expression values for 'mean.delta.ct.norm' as in above figure, with minimum and maximum value provided; this is required for *NADH-GOGAT* and *NIR* to ensure the model can distinguish the low NO_3^- and high NO_3^- despite the 'mean.delta.ct.norm' being similar in range. The x-axis is termed 'xpred' and is the predicted soil NO_3^- from actual values provided; when field data is analysed with the model it is the 'xpred' which will be deduced from the raw geometric expression data provided. Error region in light grey ribbon shows the upper and lower limit of the model, with the small the region indicating the smaller the range of error between predicted soil NO_3^- results. Details found in Section 2.1.20.



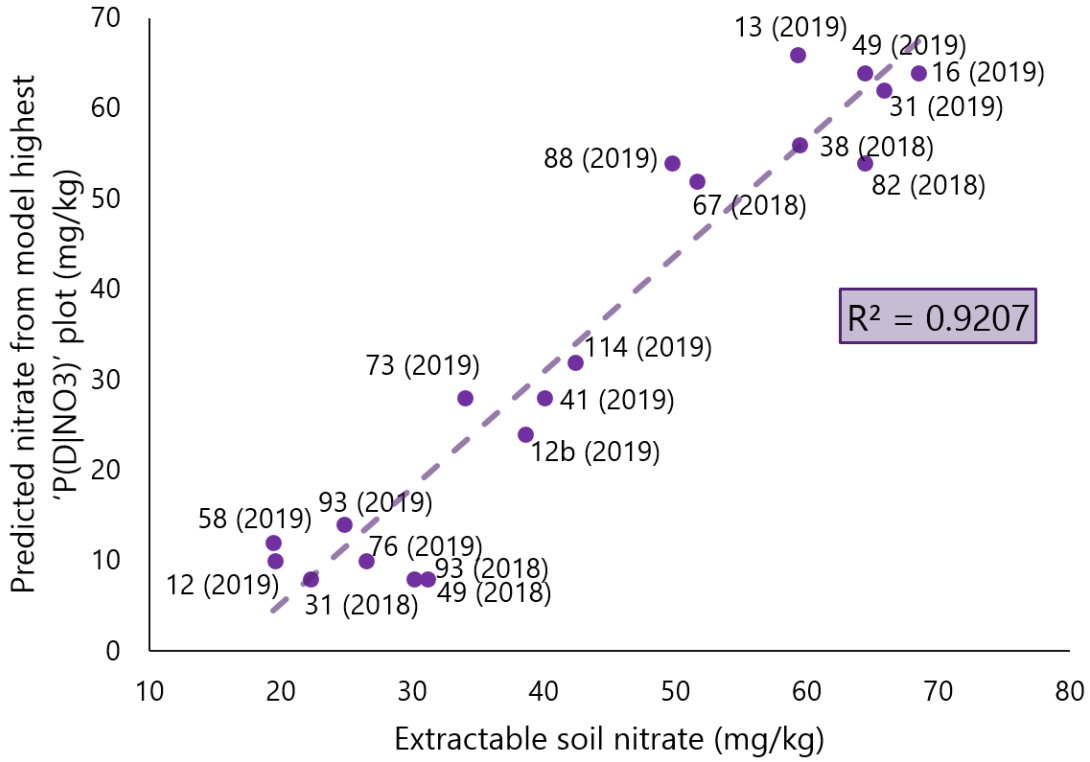
<u>Figure A10:</u> Analysis of individual gene models for 2019 field grown *Festulolium*.

Field sample expression data from 2019 was calculated in relation to two reference genes and the normalised gene expression for each gene calculated using a glasshouse tissue generated Gaussian model expression, found in Figure A5; 'yhat' is the mean values for 'mean.delta.ct.norm', and x-axis 'xpred' is predicted soil NO_3^-. Details found in Section 2.1.20.



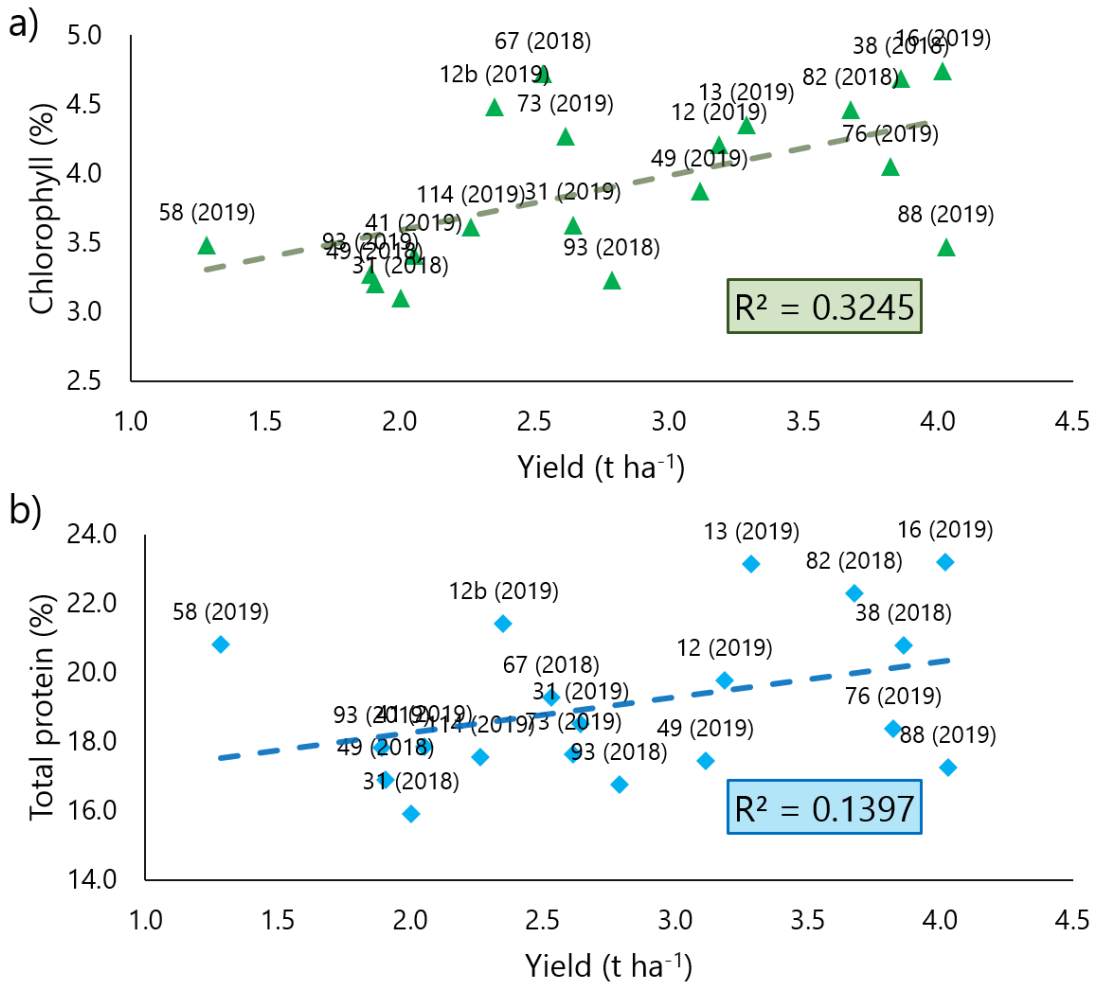
<u>Figure A11:</u> Analysis of combined gene models for 2019 field grown *Festulolium*.

Field sample combined normalised gene expression analysed with glasshouse 2019 generated Gaussian model. Here y-axis is 'P(D|NO3)' which is a symbolic derivative for simple expressions of individual gene 'xpred' values shown in Figure 3.8, thus indicating a single area of expression to relate to x. The x-axis is again 'xpred', here termed 'predicted equivalent greenhouse NO3'. This provides a visual representation of predicted concentrations in the field soil with the higher the 'P(D|NO3)' bar points the more reliable the interpretation of predicted soil NO_3^-.



<u>Figure A12:</u> Extractable soil NO_3^- for *Festulolium* fields from 2018 and 2019, correlated to model predicted soil NO_3^- from probability distribution from combined gene models generated with glasshouse data.

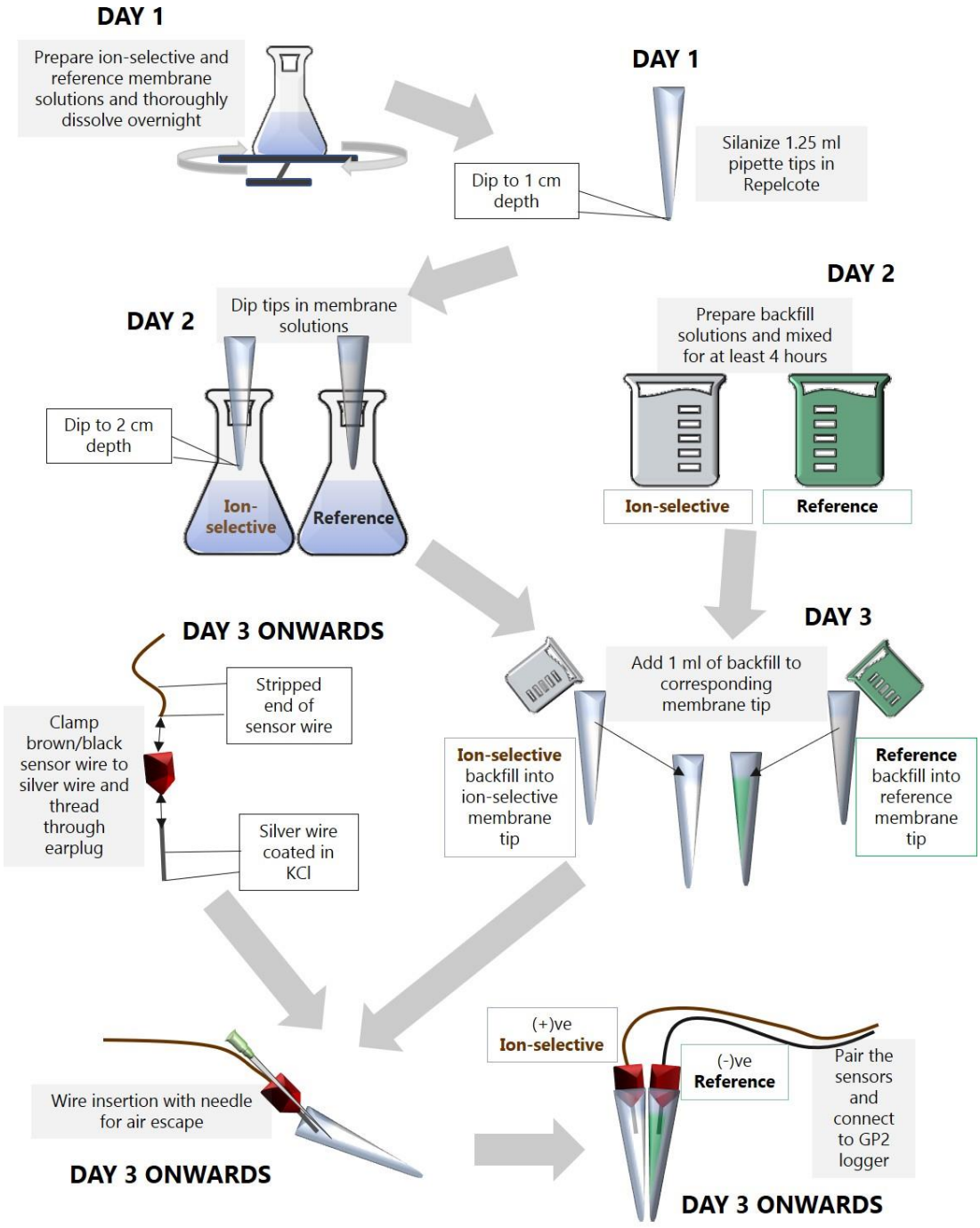
Extractable soil NO_3^- as mg/kg sampled at time of expression sampling is shown correlated to predicted soil NO_3^- based on the highest 'P(D|NO3)' bar point for each field from Figure 3.9. 'P(D|NO3)' is the estimated probability distribution across predicted greenhouse equivalent NO_3^- concentration. Linear regression is calculated as $R^2 = 0.92$.



<u>Figure A13:</u> *Festulolium* yield for fields from 2018 and 2019, with comparison to vegetative chlorophyll and total protein.

Crop yield in t ha^{-1} was measured ~ 3 weeks after gene expression sampling is shown correlated to vegetative tissue a) chlorophyll (green triangles) and b) total protein (blue diamonds) sampled at time of expression sampling. Chlorophyll and total protein extraction measured as in Sections 2.1.16 and 2.1.17. Linear regression is shown with R^2 = 0.32 for chlorophyll and R^2 = 0.14 for total protein.

Appendix B: Chapter 4 supplemental figures

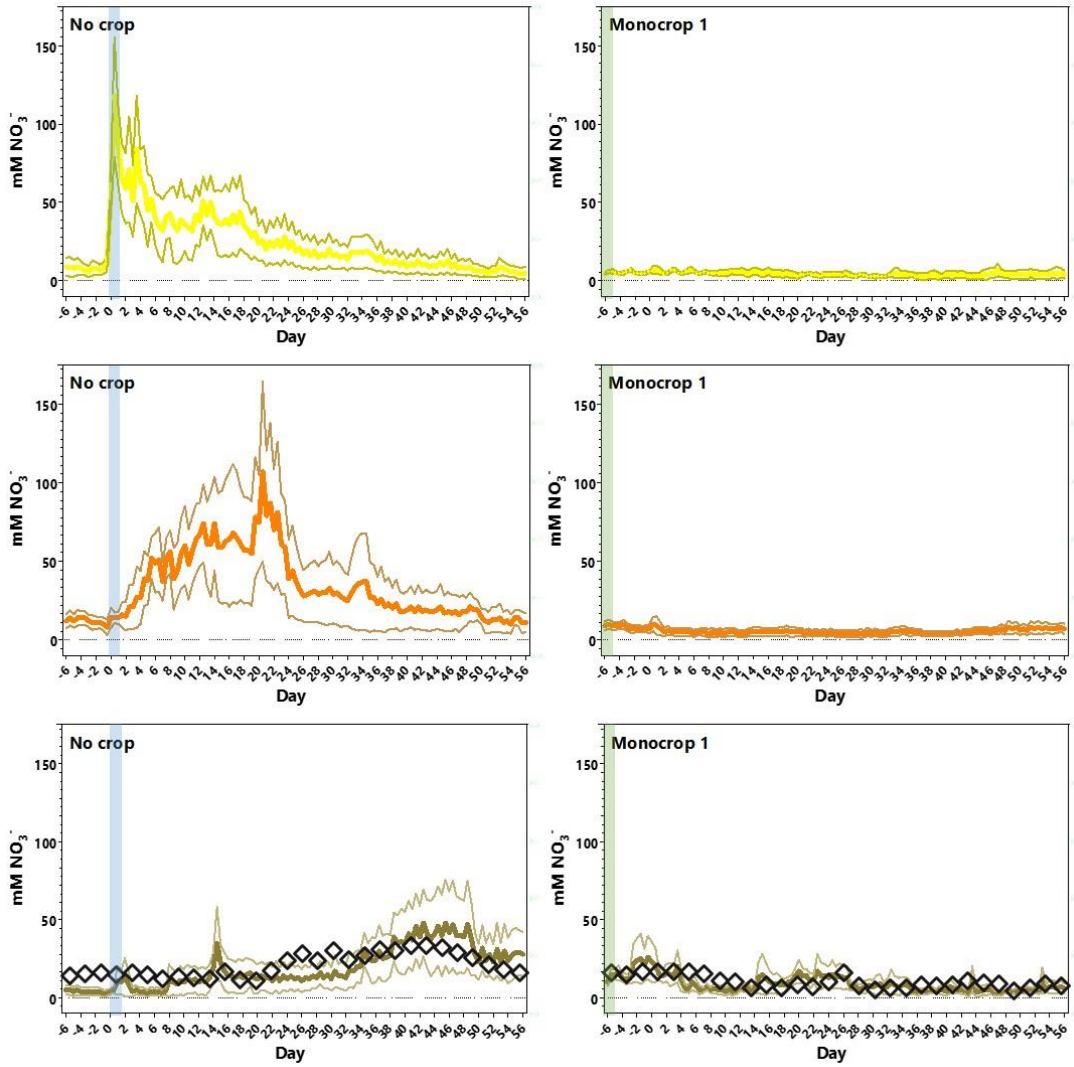


<u>Figure B1:</u> Schematic of nitrate-selective sensor construction.

Details outlined in Section 2.2.1.

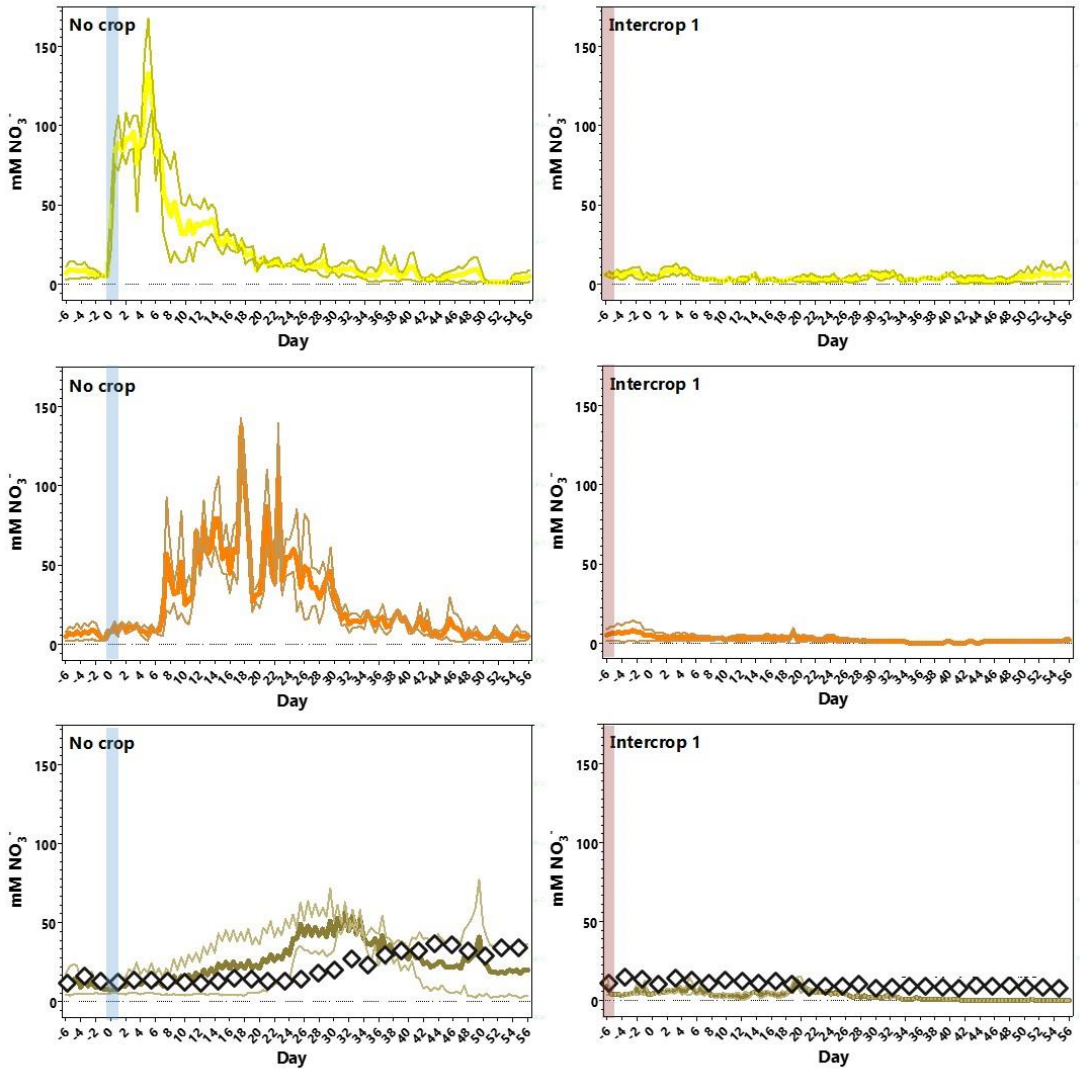
<u>Figure B2:</u> Photograph of soil column set-up for nitrate-selective sensor experiments.

Details described in Section 2.2.4.



<u>Figure B3:</u> *Lolium perenne* monocrop column experiment NO_3^--selective sensor data for 'No crop' and 'Monocrop 1'.

Column set-ups are described in Table 4.1. NO_3^--selective sensor data are shown independently for top (yellow), middle (orange), and bottom (brown) levels of columns, as described in Figure 4.3. Data is the 12-hourly average of four experimental replicates plotted in GraphPad Prism 7 (GraphPad Software Inc.), with standard errors of the means indicated with thinner lines of a similar colour. Coloured vertical bars indicate management practice of *L. perenne* crop planted (green) and nitrate application at day 0 (blue). In the bottom level graph the soil water from drainage holes for one experiment was tested as leachate using the conventional chemical assay methods described in Section 2.1.15, and indicated by black diamond symbols.



<u>Figure B4:</u> *Lolium perenne* and *Medicago sativa* intercrop column experiment NO_3^--selective sensor data for 'No crop' and 'Intercrop 1'.

Column set-up are described in Table 4.2. NO_3^--selective sensor data are shown independently for top (yellow), middle (orange), and bottom (brown) depth levels of the columns, as described in Figure 4.3. Data is the 12-hourly average of two experimental replicates, with standard errors of the means indicated with thinner lines of a similar colour. Coloured vertical bars indicate the management practices for the *L. perenne* and *M. sativa* crops (pink), nitrate application at day 0 (blue). In the bottom level graph the soil water from drainage holes from one experiment was tested as leachate using the conventional chemical assay methods described in Section 2.1.15, and indicated by black diamond symbols.

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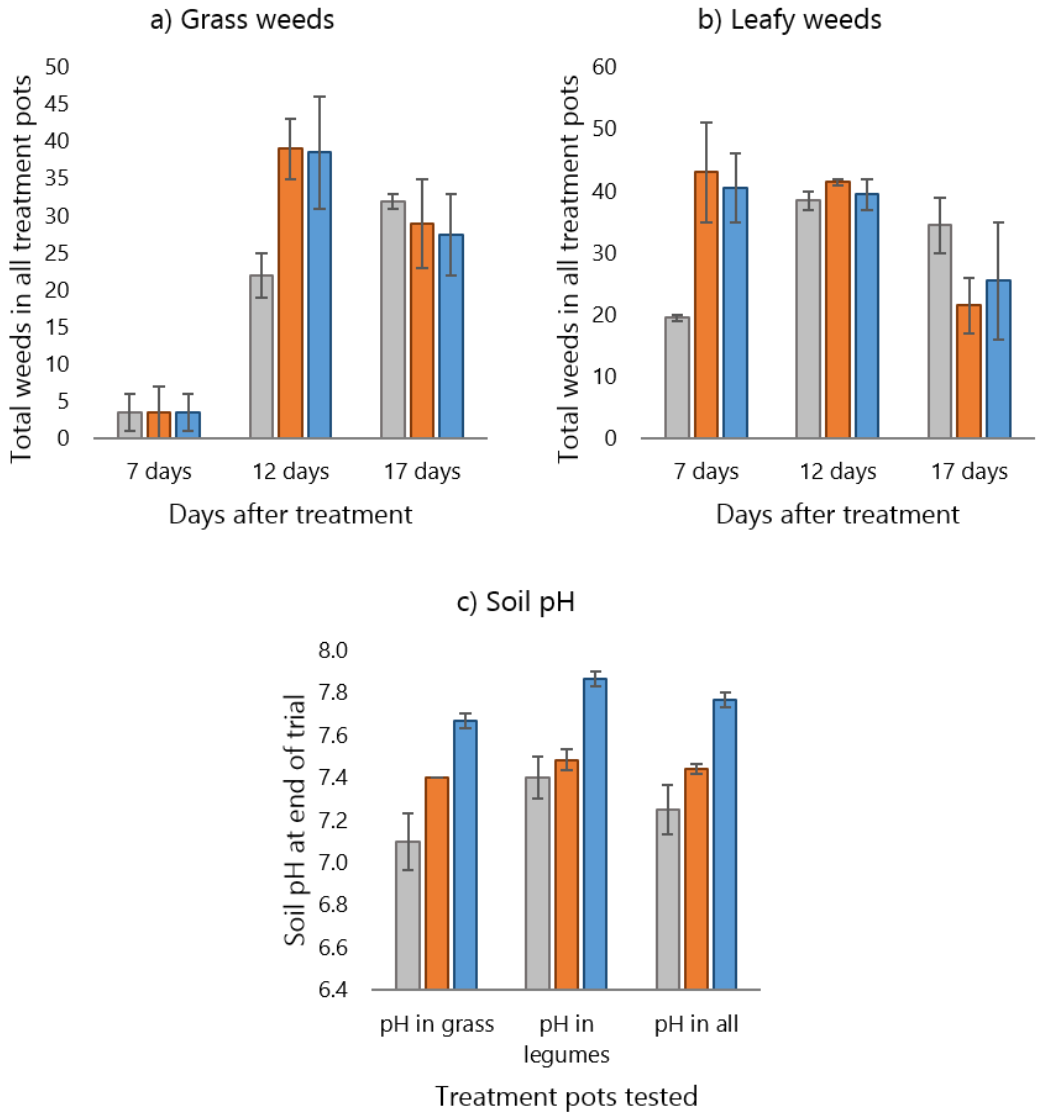
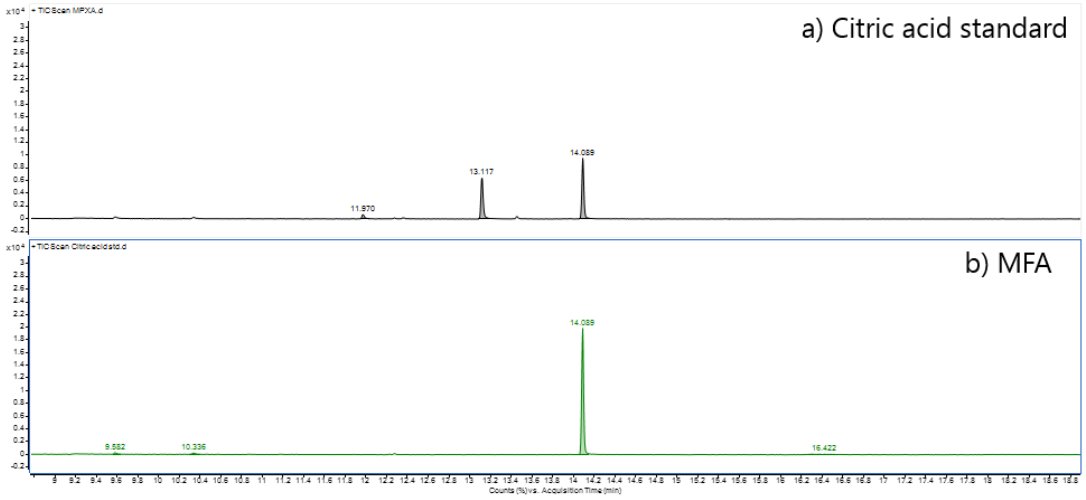
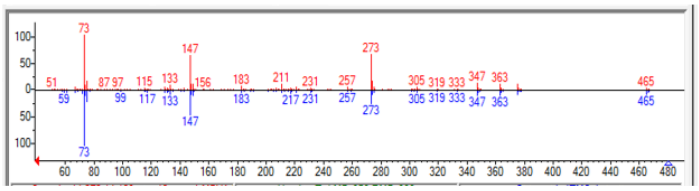
Appendix C: Chapter 5 supplemental figures



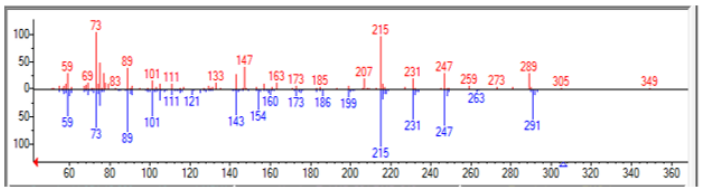
<u>Figure C1:</u> Weed counts and soil pH of pots with forage crops following one of two fulvic acid treatments relative to a control.

Treatments were deionised water (dH_2O in grey), 0.5 % MFA (MFA in blue), or 1 % VFA (VFA in orange). Treatments were applied to seedlings at 7 days post germination and weed counts were assessed at 7, 12, and 17 days post treatment in every pot. Grass weeds (a) were those identified as couch grass or similar, and leafy weeds (b) were those such as buttercups, nettles, chickweeds, or similar dicot seedling. Total weeds were calculated for all treatment pots. Soil pH was tested at the end of the experiment, with soil tested from treatment pots of six different forage species, three grass species and three legume species. Average measurements for two independent experiments are shown in bar charts above. Error bars show standard error between experiments.





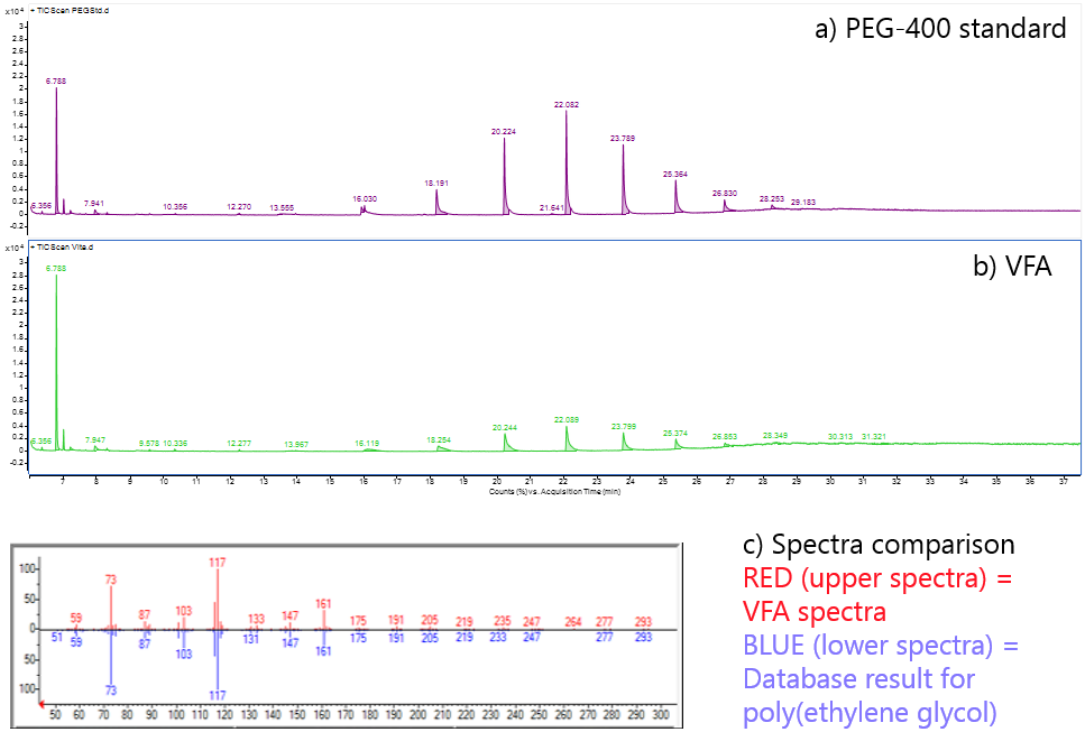
c) Spectra comparison
RED (upper spectra) = MFA spectra
BLUE (lower spectra) = Database result for citric acid (4TMS derivative)



d) Spectra comparison
RED (upper spectra) = MFA spectra
BLUE (lower spectra) = Database result for citric acid (Trimethyl TMS derivative)

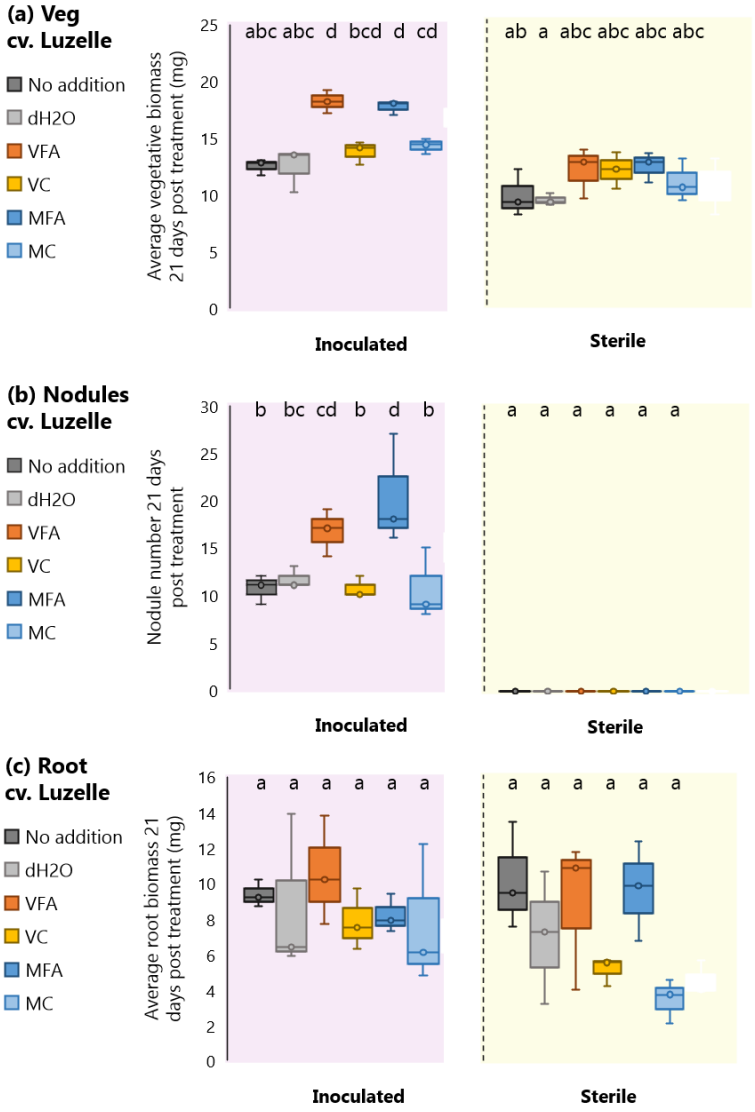
<u>Figure C2:</u> Gas chromatogram spectra of (a) citric acid standard and b) MFA, with c)-d) NIST Atomic Spectra database 1A v14 matches for citric acid, 4TMS derivative and Trimethyl TMS derivative.

GC-MS was performed as in Section 2.3.8, with both citric acid monohydrate standard and MFA at a concentration of 0.01 g/mL. The most abundant peak (14.089) was clearly citric acid, when compared with standard and tested by NIST Atomic Spectra database [162]; c) and d) show high similarity to library entries for two derivatives of citric acid, 93.3 % and 19.8 % respectively. Different derivatives of other citrates may have been due to chelation with metals with mass loss of metals during the experiment. The abundance peaks gave an accounted mass of MFA:citric = 7.2:10. Experiment performed by Freddie Morrison under supervision of Paul Brett, with graph by N.C.



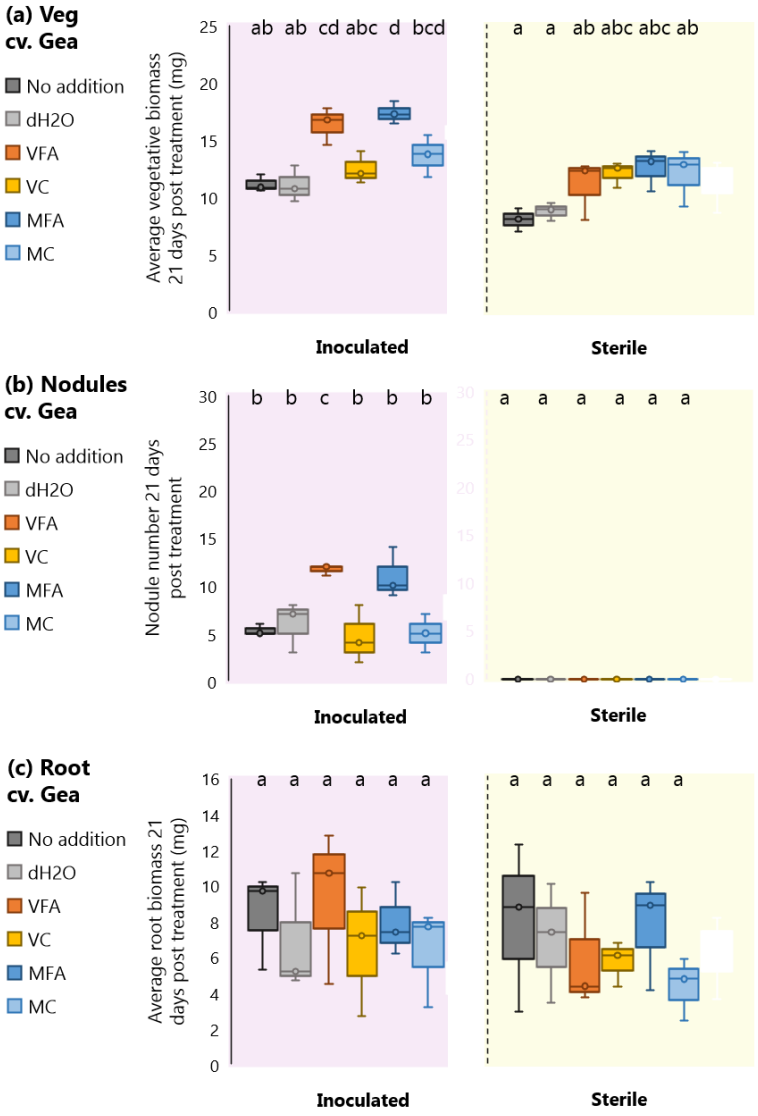
<u>Figure C3:</u> Gas chromatogram spectra of (a) PEG-400 standard and b) VFA, with c) NIST Atomic Spectra database 1A v14 matches for poly(ethylene glycol) (heptaethylene glycol).

GC-MS was performed as in Section 2.3.8, with both poly(ethylene glycol)-400 standard and VFA at a concentration of 0.01 g/mL. The staggered peaks across chromatogram was clearly a poly(ethylene glycol), when compared with standard and tested by NIST Atomic Spectra database [162]; c) show high similarity to library entries for poly(ethylene glycol) (heptaethylene glycol) of 93.43 %. The abundance peaks gave an accounted mass of VFA:PEG = 6.9:10. Experiment performed by Freddie Morrison under supervision of Paul Brett, with graph by N.C.



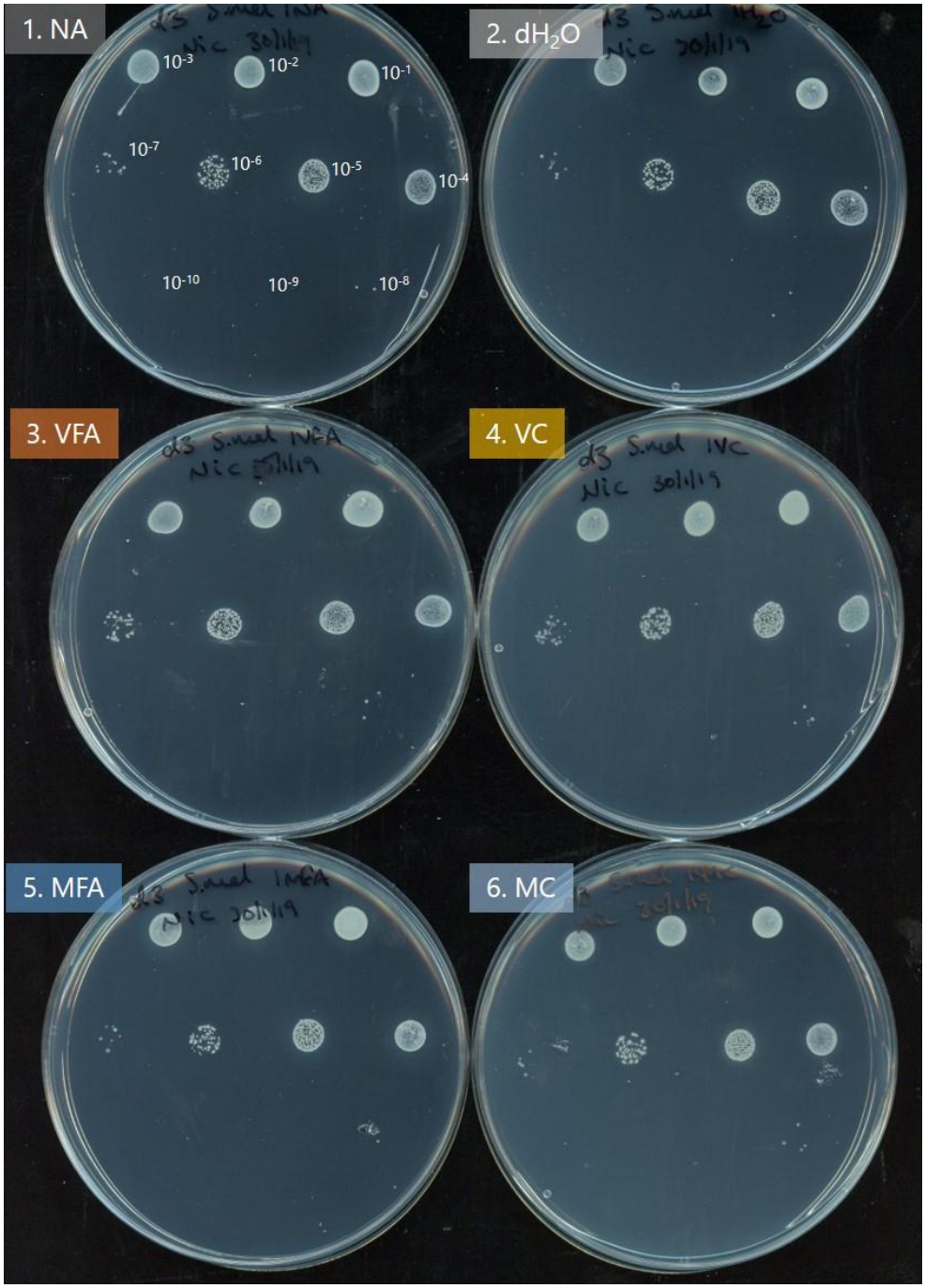
<u>Figure C4:</u> Vegetative and nodule phenotypes of *Medicago sativa* cv. Luzelle following treatment with fulvic acids or elemental controls, with or without inoculation of *Sinorhizobium meliloti.*

Two day old seedlings were transferred to media plates containing treatments as follows; no addition (NA in dark grey); deionised water (dH_2O in grey); 0.5 % MFA (MFA in blue); 0.5 % MC (MC in light blue); 1 % VFA (VFA in orange); 1 % VC (VC in yellow). Plates either remained sterile (open dots on yellow background) or inoculated with *Sinorhizobium meliloti* (closed dots on purple). At 21 days their nodule numbers were counted, and biomass determined for both vegetative tissue and full root tissue. Five seedlings were measured for each treatment condition, and total measurements for three independent experiments are shown in charts. Multiple comparisons were conducted using a one-way ANOVA Tukey test shown with letters, with significance level of 0.05.



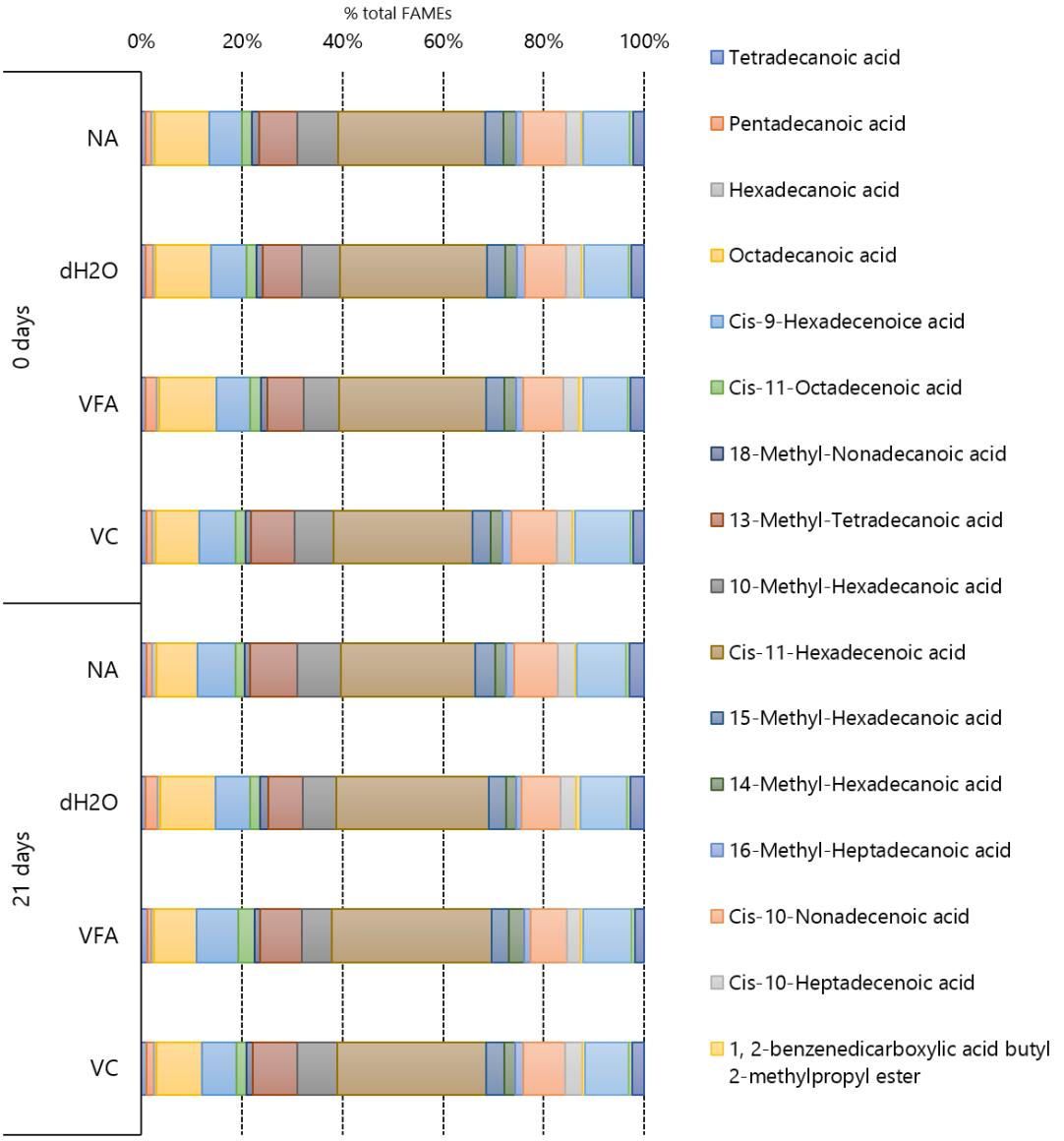
<u>Figure C5:</u> Vegetative and nodule phenotypes of *Medicago sativa* cv. Gea following treatment with fulvic acids or controls, with or without inoculation of *Sinorhizobium meliloti.*

Two day old seedlings were transferred to media plates containing treatments as follows; no addition (NA in dark grey); deionised water (dH_2O in grey); 0.5 % MFA (MFA in blue); 0.5 % MC (MC in light blue); 1 % VFA (VFA in orange); 1 % VC (VC in yellow). Plates either remained sterile (open dots on yellow background) or inoculated with *Sinorhizobium meliloti* (closed dots on purple). At 21 days their nodule numbers were counted, and biomass determined for both vegetative tissue and full root tissue. Five seedlings were measured for each treatment condition, and total measurements for three independent experiments are shown in charts. Multiple comparisons were conducted using a one-way ANOVA Tukey test shown with letters, with significance level of 0.05.



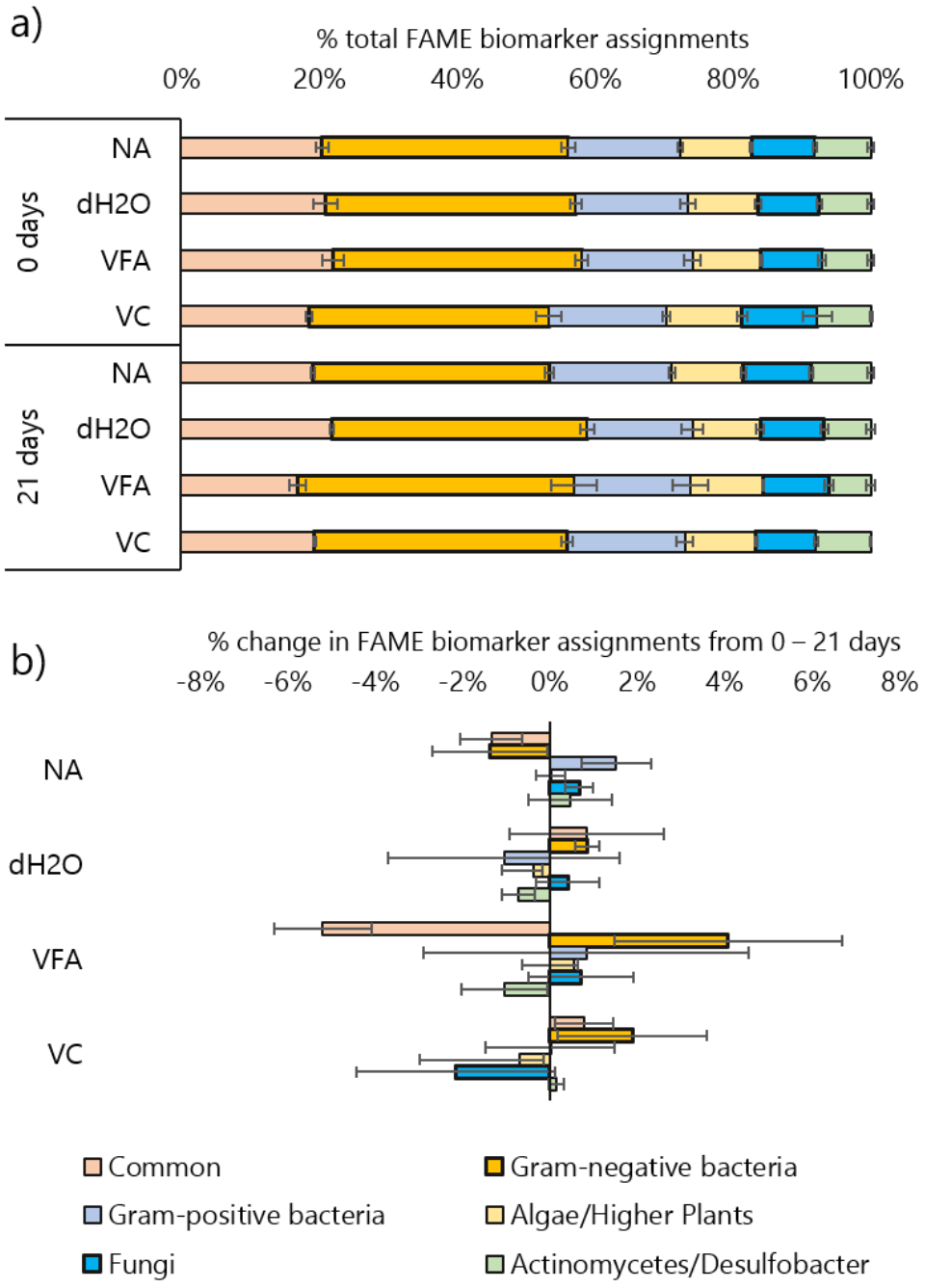
<u>Figure C6:</u> CFU counting of *Sinorhizobium meliloti* for growth effects of fulvic acid in TY medium, compared to elemental controls.

TY cultures containing treatments as follows were inoculated with *Sinorhizobium meliloti*; no addition (NA in dark grey); deionised water (dH_2O in grey); 0.5 % MFA (MFA in blue); 0.5 % MC (MC in light blue); 1 % VFA (VFA in orange); 1 % VC (VC in yellow). Average colony forming unit (CFU) counts were obtained from triplicate samples on 0 - 4 days of incubation with shaking 220 rpm at 28 °C. The above example is from 3 days.



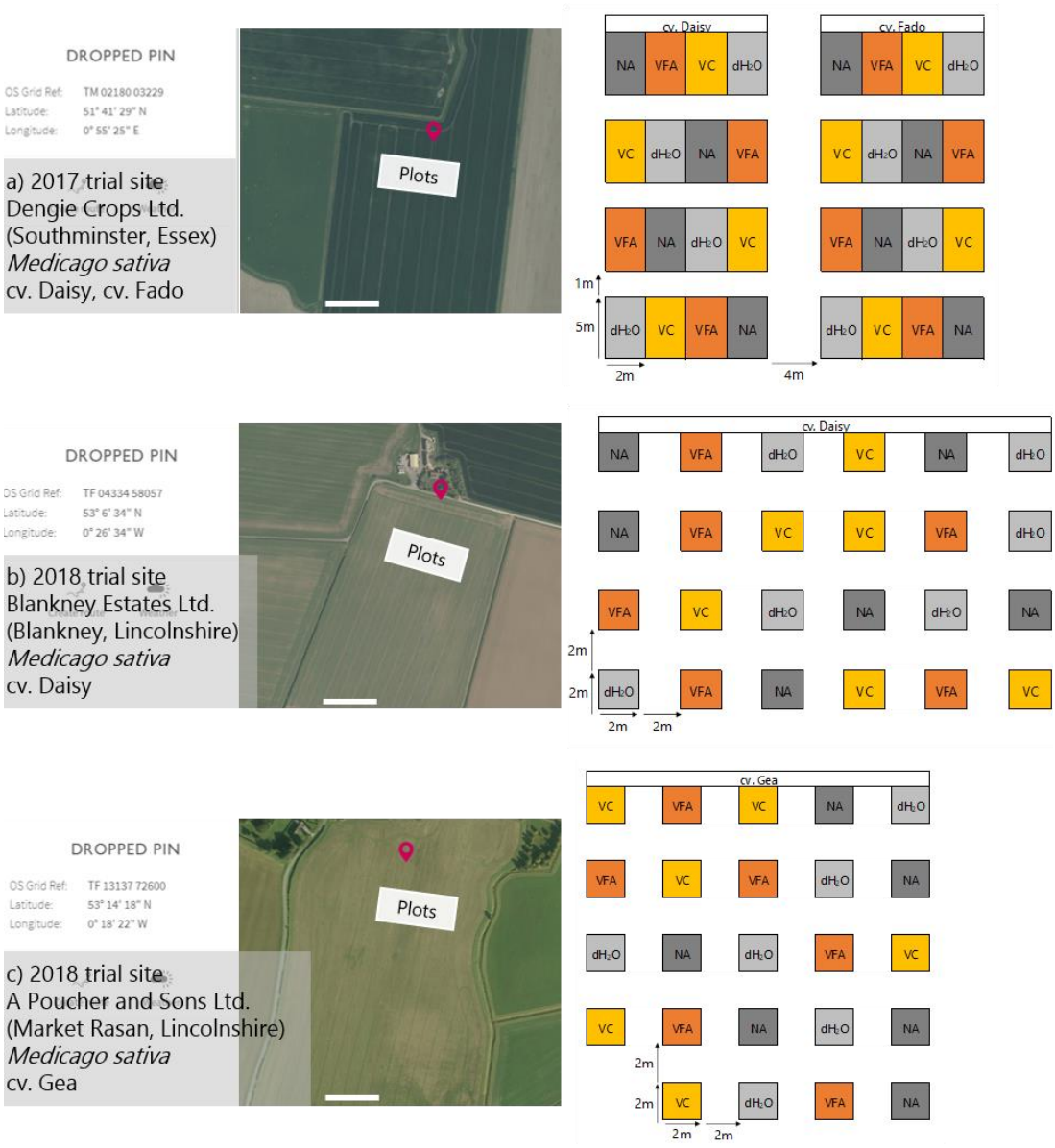
<u>Figure C7:</u> Fatty acid methyl esters (FAMEs) relative contents from soil treated with fulvic acid or controls.

Soil from *Medicago sativa* assays with fulvic acid applications (NA; dH_2O; VFA; VC) was extracted for PLFA content at 0 days and 21 days, for three separate experiments, and converted into FAMEs. Individual FAME relative abundance (in % of total FAMEs) was calculated from total FAME biomass (nmol g^{-1} of dry soil), as shown above.



<u>Figure C8:</u> Fatty acid methyl esters (FAMEs) biomarker assignments to microbial popoulation relative contents from soil treated with fulvic acid or controls.

Soil from *Medicago sativa* assays with fulvic acid applications (NA; dH_2O; VFA; VC) was extracted for PLFA content at 0 days and 21 days, for three separate experiments, and converted into FAMEs. Individual FAME relative abundance (in % of total FAMEs) was calculated from total FAME biomass (nmol g^{-1} of dry soil). FAMEs were associated with organism as in literature [166], as shown above with a) relative total FAME biomarker assignment for each treatment on both days, and b) the change in FAME biomarkers between 0 and 21 days for each treatment.

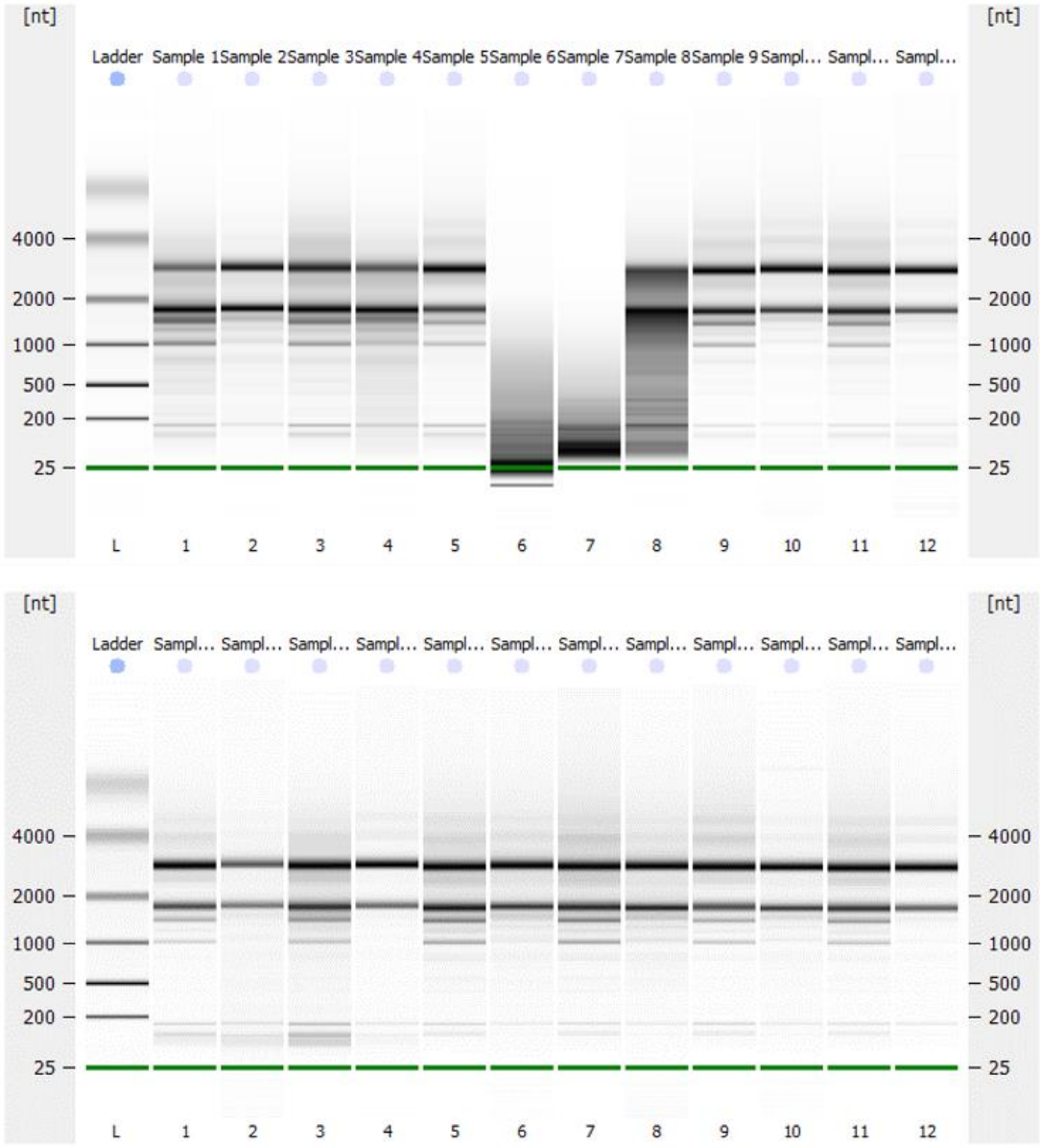


<u>Figure C9:</u> Field plots for fulvic acid trials performed in 2017 and 2018. Treatments were applied to field plots at beginning of establishment and vegetative yields were assessed before 1st cut of growing season. Plot areas are provided, and treatments were; no addition (NA in dark grey); deionised water (dH_2O in grey); 1 % VFA (VFA in orange); and 1 % VC (VC in yellow). In 2017 trials were performed at Dengie Crops Ltd. (Southminster, Essex) with four plots per treatment of both cv. Daisy and Fado, shown in a). In 2018 the trials were at both Blankney Estates Ltd. (Blankney, Lincolnshire) and A Poucher and Sons (Bardney Dairies) Ltd. (Market Rasan, Lincolnshire) with six plots per treatment of cv. Daisy and Gea respectively, shown in b) and c). Maps were generated using Ordnance Survey OpenData OS Open Greenspace – GB (data type: vector, supply format: GML 3): Contains OS data © Crown copyright and database rights (2019).

Appendix D: Chapter 6 supplemental figures

Table D1: RNA quality check for RNAseq samples in Section 2.4.3.

Sample no.	Treatment	Tissue	Timepoint (day)	Experiment replicate	Total amount (ng)	Concentration (ng/µL)	RIN	Nanodrop 260/280
1	Fulvic	Shoot	0	1	8295.9873	360.6951	6.2	1.88
2	Fulvic	Root	0	1	3466.6543	150.7241	7.9	N/A
3	Control	Shoot	0	1	7392.2	321.4	7.1	N/A
4	Control	Root	0	1	8167.8543	355.1241	6.7	1.9
5	Fulvic	Shoot	3	1	6739	293	8.2	N/A
6	Fulvic	Root	3	1	7221.4894	313.9778	4.2	1.98
7	Control	Shoot	3	1	6920.7	300.9	4.5	1.93
8	Control	Root	3	1	8474.0648	368.4376	6.8	1.87
9	Fulvic	Shoot	0	2	12387.8	538.6	7.7	N/A
10	Fulvic	Root	0	2	4898.2065	212.9655	9.2	N/A
11	Control	Shoot	0	2	7399.1	321.7	7.8	N/A
12	Control	Root	0	2	2078.5008	90.3696	9.5	N/A
13	Fulvic	Shoot	3	2	8795.3403	382.4061	8.8	N/A
14	Fulvic	Root	3	2	1340.52763	58.28381	9.1	N/A
15	Control	Shoot	3	2	7813.9441	339.7367	7.7	N/A
16	Control	Root	3	2	2769.3334	120.4058	10	N/A
17	Fulvic	Shoot	0	3	14812	644	7.7	N/A
18	Fulvic	Root	0	3	7420.5452	322.6324	8.7	N/A
19	Control	Shoot	0	3	7024.2	305.4	7.9	N/A
20	Control	Root	0	3	4492.9787	195.3469	8.4	N/A
21	Fulvic	Shoot	3	3	16010.3	696.1	8.9	N/A
22	Fulvic	Root	3	3	4206.9392	182.9104	9.2	N/A
23	Control	Shoot	3	3	14356.6	624.2	8.4	N/A
24	Control	Root	3	3	7120.8	309.6	9.9	N/A



<u>Figure D1:</u> Gel from Bioanalyzer 2100 run of RNAseq samples.

Details are in Section 2.4.3. Lane numbers match sample numbers in Table D1 above.

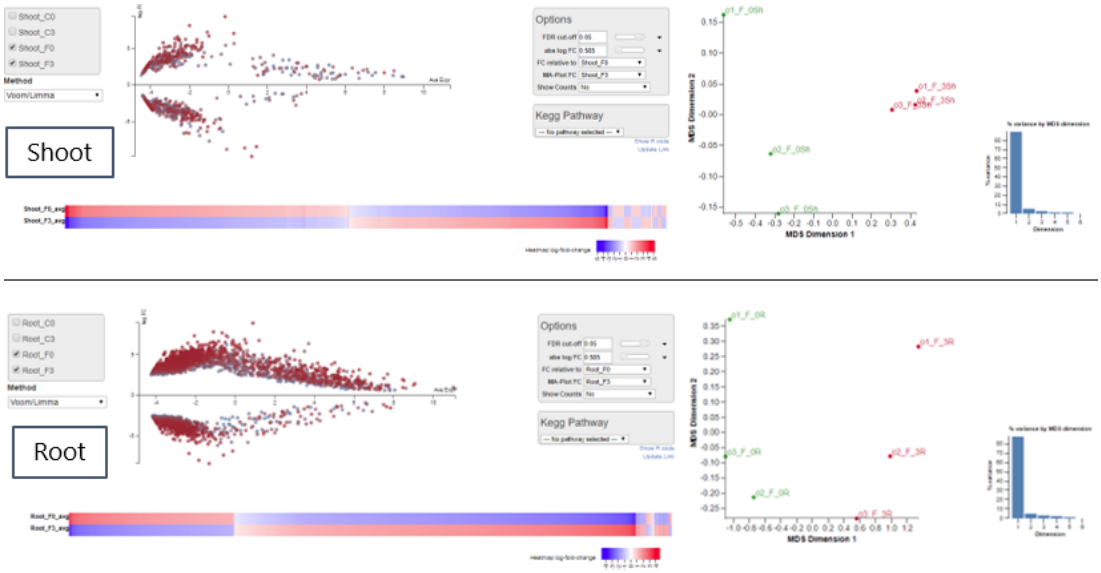
Table D2: Sequencing quality check for RNAseq samples in Section 2.4.5.

Effective Rate (%) is percentage of clean reads divided by raw reads, Error (%) is the base error rate, Q20 and Q30 relate to base count of Phred value > 20 or > 30 respectively divided by total base count and GC(%) was bases G and C divided by total count.

Sample no.	Raw reads	Clean Reads	Raw data	Clean data	Effective (%)	Error (%)	Q20 (%)	Q30 (%)	GC (%)
1	22973960	22535108	6892188000	6760532400	98.09	0.03	97.02	91.88	41.25
2	22902670	22522045	6870801000	6756613500	98.34	0.03	96.9	91.65	41.71
3	20829575	20201808	6248872500	6060542400	96.99	0.03	97.12	92.09	42.11
4	20231682	19865807	6069504600	5959742100	98.19	0.03	96.47	90.83	41.98
5	22912434	22562546	6873730200	6768763800	98.47	0.03	96.91	91.68	42.72
6	21217369	20736820	6365210700	6221046000	97.74	0.03	96.55	90.96	41.36
7	24176064	23832798	7252819200	7149839400	98.58	0.03	96.74	91.29	42.62
8	22920551	22616180	6876165300	6784854000	98.67	0.03	96.94	91.72	41.53
9	20995814	20723523	6298744200	6217056900	98.7	0.03	96.99	91.84	42.1
10	23979006	23681565	7193701800	7104469500	98.76	0.03	96.5	90.85	41.38
11	20951309	20639160	6285392700	6191748000	98.51	0.03	97.06	91.95	41.88
12	19831106	19614353	5949331800	5884305900	98.91	0.03	96.98	91.87	41.95
13	21828245	21521049	6548473500	6456314700	98.59	0.03	97.33	92.61	42.45
14	22555556	22140153	6766666800	6642045900	98.16	0.03	97.03	92	41.66
15	23481991	22987941	7044597300	6896382300	97.9	0.03	96.94	91.7	42.38
16	21101817	20618591	6330545100	6185577300	97.71	0.03	97.07	92.14	41.91
17	20483130	20160497	6144939000	6048149100	98.42	0.03	97.22	92.34	42.12
18	21714169	21311860	6514250700	6393558000	98.15	0.03	97.32	92.62	41.36
19	23035824	22710308	6910747200	6813092400	98.59	0.03	97.31	92.56	42.75
20	21097923	20501325	6329376900	6150397500	97.17	0.03	97.19	92.28	41.25
21	22941886	22582560	6882565800	6774768000	98.43	0.03	97.09	92.09	42.97
22	22043314	21640054	6612994200	6492016200	98.17	0.03	96.88	91.66	41.9
23	23621835	23184386	7086550500	6955315800	98.15	0.03	97.19	92.22	42.3
24	19159745	18843797	5747923500	5653139100	98.35	0.03	97.06	92.11	41.98

Table D3: Pseudoalignment summary of RNA-seq samples from de novo transcriptome analysis in Section 2.4.5.

Sample no.	Treatment	Tissue	Timepoint (day)	Experimental rep	Reads	Reads pseudoaligned	% pseudoaligned
1	Fulvic	Shoot	0	1	21948373	15001567	68.35
2	Fulvic	Root	0	1	21683448	15322780	70.67
3	Control	Shoot	0	1	19731245	13433374	68.08
4	Control	Root	0	1	19192851	13111977	68.32
5	Fulvic	Shoot	3	1	21916558	13940709	63.61
6	Fulvic	Root	3	1	19970525	14133478	70.77
7	Control	Shoot	3	1	23125595	14603288	63.15
8	Control	Root	3	1	21818824	15337857	70.30
9	Fulvic	Shoot	0	2	20015180	13166950	65.78
10	Fulvic	Root	0	2	22756103	15965816	70.16
11	Control	Shoot	0	2	20017217	13281556	66.35
12	Control	Root	0	2	18832338	13193369	70.06
13	Fulvic	Shoot	3	2	20834945	13749556	65.99
14	Fulvic	Root	3	2	21347709	15176499	71.09
15	Control	Shoot	3	2	22410937	14604137	65.17
16	Control	Root	3	2	19940906	14437150	72.40
17	Fulvic	Shoot	0	3	19550309	12674614	64.83
18	Fulvic	Root	0	3	20587135	14609678	70.97
19	Control	Shoot	0	3	22060176	13759703	62.37
20	Control	Root	0	3	19868638	14429523	72.62
21	Fulvic	Shoot	3	3	21961363	14221382	64.76
22	Fulvic	Root	3	3	20853270	14884900	71.38
23	Control	Shoot	3	3	22566458	15036502	66.63
24	Control	Root	3	3	18121577	12993129	71.70
				Total	501111680	341069494	-
				Mean	20879653.3	14211229	68.15



<u>Figure D2:</u> Differential transcript expression using voom/Limma method [184] between 0 day (green) and 3 day (red) for VFA treatment RNA samples (see 2.4.1) using Degust [183] as in Section 2.4.6.

Shoot and root tissue are analysed seperately and transcripts are only deemed differentially expressed if all experimental replicates have an absolute log fold change of 0.585 and false-dscovery q-value < 0.05.

Appendix E: ‘UK dried forage production: a review of industry changes and assessment of prospects for both policy and science’

E.1 GENERAL INTRODUCTION

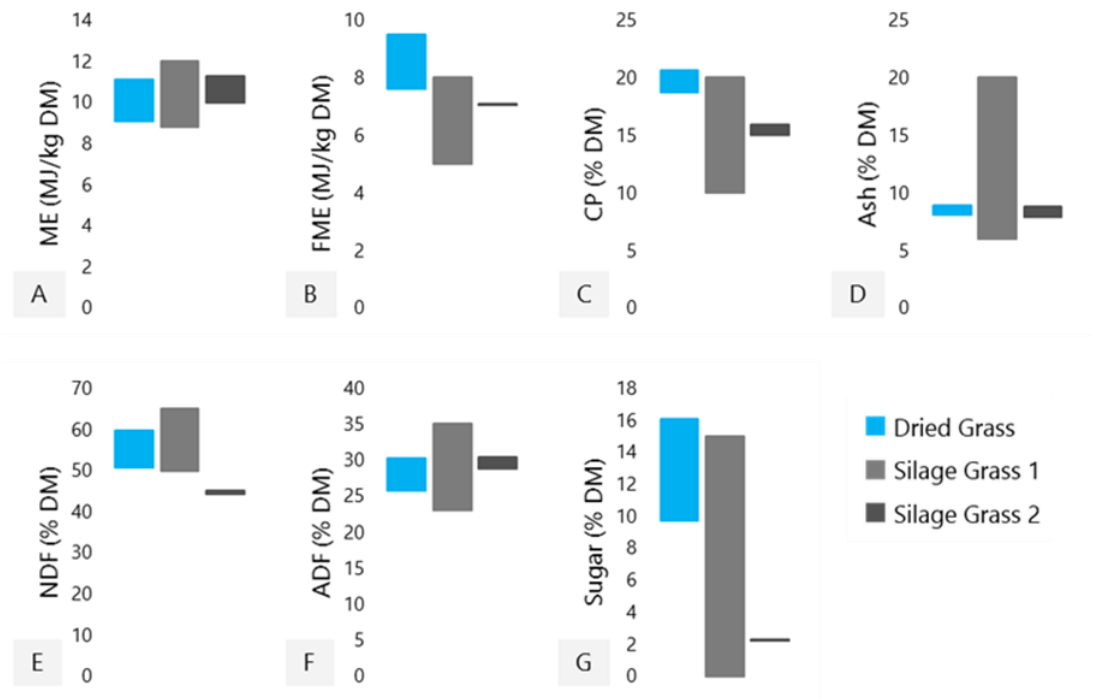
In the UK, dried forage has been produced since the 1930s, with most producers being members of the British Association of Green Crop Driers (BAGCD) [8]. The BAGCD is an association of numerous farms based in Cambridgeshire, Devon, Essex, Hertfordshire, Lincolnshire, Perthshire and Yorkshire. In total they farm approximately 7500 hectares of land across all these sites, with only one farm as permanent pasture, with total production worth ~ £8 – 9 million per annum [486]. The fundamentals of the grass-drying process arose at Cambridge between 1925 – 1927 [61-63] and as a general description these producers grow various grass and herbaceous legume forage species, both stand-alone or in mixed cropping; Table E1 below shows those crops cultivated by BAGCD at present, and those forage crops not currently grown, but which either were historically grown or may be in the future.

Forage crops are grown across the standard UK growing season and are cut to be high temperature dried in large rotary drum dryers at ~ 800 °C; unlike in hay or silage production swaths, which are left to wilt post-cutting for only a few hours before being high temperature dried to a moisture content of 12 %. The dried product, also termed 'Dehy' due to its dehydration, is then milled and pelleted, and used in cattle, equine, poultry, and pet feeds.

The nutritional value of dried forage has similar nutritional content to silage forage, but as it is in a dry, pelleted form, these nutritional levels are retained for longer. Figure E1 shows the ranges for different nutritional parameters of BAGCD produced dried grass, along with two independent ranges for silage grass. One can see for each parameter the nutritional composition is similar for all, however dried forage can be kept for many years with low nutritional changes [64] compared to some silage forage, which over only 6 months can have quality changes in Crude Protein (CP), Neutral Detergent Fibre (NDF), and Acid Detergent Fibre (ADF) [828].

Table E1: Forage crops currently cultivated by BAGCD members across the UK, or those not cultivated but popular for other forage crop growers. These are grouped into either grass or legumes, with the species and common name included. cultivars being drilled shown for the most widely sown species.

Current crops cultivated by BAGCD			
Grasses		**Legumes**	
Species	**Common name**	**Species**	**Common name**
Lolium perenne	Perennial Ryegrass	*Medicago sativa*	Lucerne/Alfalfa
Festuca arundinacea	Tall Fescue	*Lotus corniculatus*	Birdsfoot Trefoil
Festuca pratensis	Meadow Fescue	*Trifolium pratense*	Red Clover
***Festuca* x *Lolium* (various)**	*Festulolium*	*Pisum sativum*	Spring Pea
Phleum pratense	Timothy grass		
Anthoxanthum odoratum	Sweet Vernal grass		
Crops of interest to BAGCD			
Grasses		**Legumes**	
Species	**Common name**	**Species**	**Common name**
Dactylis glomerata	Cocksfoot	*Onobrychis viciifolia*	Sainfoin
Lolium x boucheanum	Hybrid ryegrass	*Trifolium repens*	White Clover
Lolium multiflorum	Italian Ryegrass	*Galega orientalis*	Forage Galega
Lolium westerwoldicum	Westerwold Ryegrass		



<u>Figure E1:</u> Typical nutritional value of different processed forage grass, dried grass (blue) [64] and two examples of silage grass (grey) [829; 830].

Bars are the typical ranges for each of the following nutritional parameters in either MJ/kg Dry Matter (DM) or % DM; (A) Metabolisable Energy (ME); (B) Fermentable Metabolisable Energy (FME); (C) Crude Protein (CP); (D) Ash; (E) Neutral Detergent Fibre (NDF); (F) Acid Detergent Fibre (ADF); (G) Sugar.

E.2 HISTORICAL CULTIVATION IN THE UK

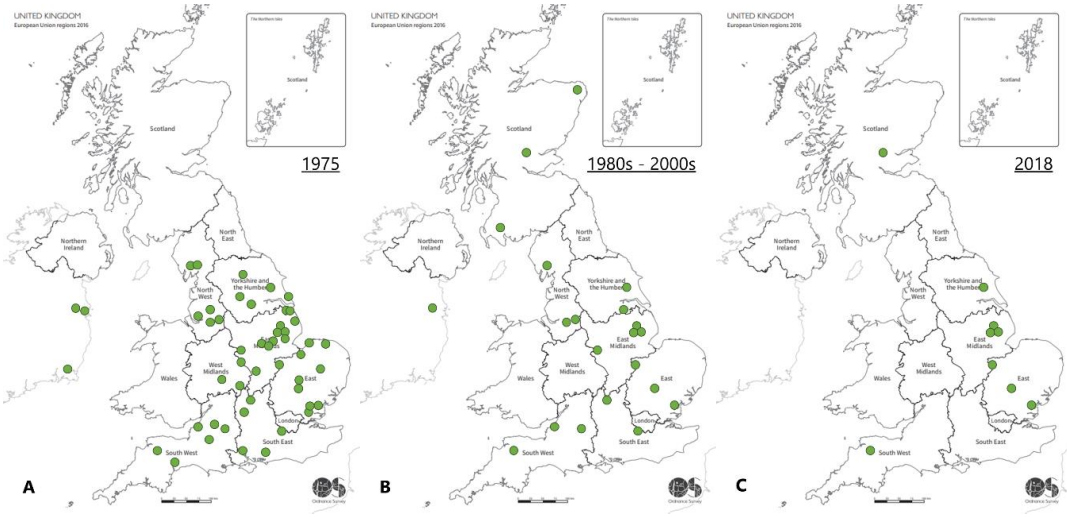
The use of wild forage pastures around 9000 – 7000 BC coincided with the domestication of animals across Europe, Asia, and North Africa [6]. Developments in haylage production, along with both forage crop domestication from ~ 1000 BC onwards and forage cultivation globally, enabled the rise of the agricultural intensification period of the 13th – 19th Centuries. This increase in agricultural intensification was particularly prominent throughout the UK [7; 6; 18].

In the 1920s the technology for drying forage crops was developed [61; 62], and in the 1930s dried forage became available for animal production [63]. As forage crop cultivation is intrinsic to intensive agriculture, it is in turn essential for supporting a growing population. The process of high temperature drying enables the feed from dried forage to last longer than normal fresh fodder, haylage or silage but retain its nutritional content (as in Figure E1 above). Therefore, high temperature drying contributes to larger scale farms with high feed demands from increased livestock numbers, increasing final output efficiencies for the population. This was particularly evident in a post-World War II push to produce efficient, low waste animal feed that could be stockpiled if necessary in future decades with proclamations "the drying of grass, lucerne and other crops will rapidly become a corner-stone of British Agriculture" [65]. From the 1950s to 1970s, across the UK forage growers were taking advantage of the new drying technology and buying rotary machines such as in Figure E2, making the industry big business. During this time a global association was set up for forage driers called the Commission Intersyndicale des Déshydrateurs Européens (CIDE) of which BAGCD is a member, and international conferences under the title of International Green Crop Drying Congress (IGCDC).

<u>Figure E2:</u> Assorted crop drying advertisements from 1970s [831; 832], showing the huge gain of interest in the agricultural techniques first developed from 1925 [62].

By 1975, the UK forage drying industry was widespread, as shown in Figure E3A, with the location of BAGCD members with active driers. Such a trend was occurring internationally with the 1973 First IGCDC having over 300 delegates in attendance from 23 countries, including many across Western Europe, but also North America and New Zealand [831]. This period was marked by increased animal product prices as social demand was high in the UK population, but agriculture would increase efficiencies required to keep up with demand until 1980s.

More efficient agricultural practices were developed throughout the century ranging from crop rotation and weed control, to confined animal feeding; these practices would eventually combine into very low-priced products. This type of farming is now termed intensive agriculture, due to the development of organic- and extensive- farming culture in the latter half of the century [833]. Consequently, as animal feed demand was high this meant "the feeding of dried green crops could play an important role [and] Nowhere is this more marked than within the 9 countries of the European Economic Community" and so demand for drying remained high [831; 832]. The only glimpse of negativity at the 1973 Congress was fuel supply was becoming a costly problem for agriculture, but as this was global and effecting all industries it was not hugely discussed.



<u>Figure E3:</u> UK and ROI forage growers and driers belonging to the British Association of Green Crop Driers (BAGCD) in (A) 1975 [832], (B) 1980s - 2000s [4; 834; 398], and (C) 2018 [8; 398].

Maps were generated using Ordnance Survey OpenData OS Open Greenspace – GB (data type: vector, supply format: GML 3): Contains OS data © Crown copyright and database rights (2018). BAGCD member sites were estimated and marked onto maps with Microsoft PowerPoint.

Towards the end of 1970s, the principle of rising energy costs was starting to worry forage crop driers. Moreover, factors such as land use disagreements, newly refined production and storage of haylage, silage, and also the use of cereal supplements for livestock, were also cause for concern [835]. However, the forage drying industry continued with the assistance of the Common Agricultural Policy (or CAP) from 1962 which gave subsidies to the six founding European Economic Community (EEC) Member States, and in 1973 the UK joined, which was later to become the European Union (EU) in 1993. The CAP is financed through national payments by member states through both the European Agricultural Guarantee Fund (EAGF) and European Agricultural Fund for Rural Development (EAFRD), supporting market changes and rural development respectively [836]. The CAP meant during this time crop driers would get approximately €66 per hectare, and these subsidies would be based on the crops per hectare/animals per head that a farm produced, but without a need for the crop in question to be in demand, and not a commodity crop like wheat. This meant that regardless of worries of the forage crop driers,

European CIDE members (including BAGCD) were guaranteed a minimum price for their work every year irrespective of the market and costs. By 1989, CIDE involvement was down to 14 countries, but there was strong feeling that the industry would continue to grow, especially the lucerne market, as "there was huge investment in some 300 drying plants, with the aim of turning 'our' wonder protein plant, lucerne, into a marketable product" [4]; Figure E3B shows a decline in driers in the UK with fewer BAGCD members, despite it still being a relatively lively industry internationally.

From the 1990s onwards, reorganisations of the CAP would severely affect UK Crop Driers. The 1992 "MacSharry Reforms" and the millennium report "Agenda 2000" were the beginning of lowered subsidies to farmers, with increased or new demands for food safety, animal welfare, and the protection of environment including biodiversity and limiting leaching of fertilisers [837]. However, it was not until 2003 when the European Commission (EC) began to reform the entire system by removing coupled subsidies altogether and instead having a single farm payment, whereby industries with high input fuel demands, like crop drying, would be most affected [838]. The EC began to look at the average three-year activity of a farm, which would be the basis for its farm single payments. Farms had to keep up with regulations to receive their payment, which meant growing competitive crops. As forage crops are not commodity crops and therefore not as competitive, their farm single payments dropped significantly when compared to their original CAP subsidies. It was necessary to ensure that UK growers were setting a strong case for the new regulations. From 1992 onwards BAGCD amended their memorandum principles to "encourage the improvement of the fertility of the soil, the betterment of grassland, and the home production of grass and dried green fodder…for the benefit of the community at large" [834]. These amendments also aligned with the sustainable farming practices that the EC was also trying to implement.

By 2005 more changes had occurred due to the Secretary of State's Guidance for Vegetable Matter Drying Processes 2005 Crown Report [839], which further affected UK growers. The initial plan from the Crown Report was to abolish all the specific subsidies that UK growers received for both

growing and producing dried fodder. However, as BAGCD had already stated clear principles for sustainable farming practices, a compromise between the industry and government was sought. It was agreed the government would divide the aid between the farms and the crop driers, with halve going to the farm payment and the other half to the crop driers. This meant BAGCD members had to have EU spot checks on quality of fodder, which if not of a high enough standard, would disallow or reduce their subsidies. Occasionally, there were problems where if the regulations were implemented literally, then they would negatively affect the feasibilities of UK forage farming. For example, under EU law growing grass for five years becomes permanent pasture, and the BAGCD was worried about this definition so negotiations were required.

The key issues surrounding these laws are the UK-specific changes to Nitrogen (N) fertiliser usage, and apart from these changes, there have been limited deviations from common agricultural law in the UK dried forage industry. Similar crops have been grown, as in Table E1, and the largest recent change has been in the 2013 CAP reform [840]. With this reform there was more emphasis on environmental issues such as provisions to stop mono-cropping. Rules that enforce requirements for producers with a big enough farmed area to grow at least 3 crops, together with the provision a farmer's largest crop cannot occupy more than 75 % of their total land were set in place. As before, this kind of legislation is sometimes not harmonious with a streamlined production process, however as shown in Table E1, BAGCD members grow a variety of species. There was a trend towards more growth of lucerne as the equine feeds industry developed. The remaining farms use crop rotations of forage crops along with break crops of wheat, maize or oil seed. Cuts are taken throughout the spring/summer growing season every 6 – 8 weeks (usually 4 – 7 cuts depending on weather conditions and growth).

Nonetheless, as can be seen in Figure E3C, the number of UK forage drying sites has been decreasing. The main reason for a drop off in production is due to European competition, however increases in fuel prices, restrictions to N fertiliser applications, and implementation of biodiversity, greening, and health and safety policies are contributing factors [398]. UK fodder is

expensive to produce, with the French and Dutch controlling much of the market. Moreover, a drop off in CIDE numbers, due to European growers not seeing benefits from membership, has left a lot of the once thriving international industry disconnected. Forage cropping, of which the drying industry is an important asset, still accounts for a substantial proportion of EU agricultural output, as shown in Figure E4. Again, this is due to its importance in animal production, which is set to continue growing globally [841]. The future of UK forage drying is unsure, and its prospects especially in terms of EU politics will be discussed later.

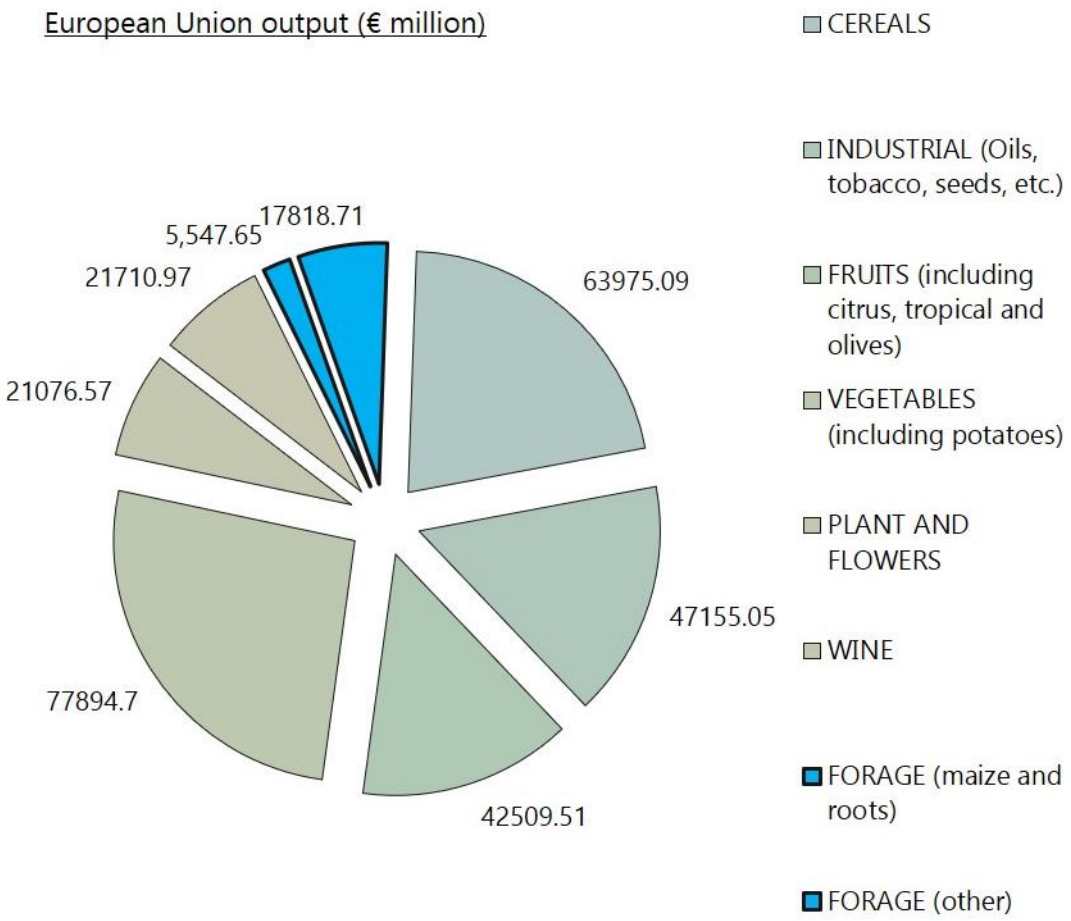


Figure E4: Total European Union output for all crops in 2016 from Eurostat data [841].

This includes a substantial portion for forage crops (bright blue), comparable to the production of fruit and wine crops, and bioenergy crops if sub-split from 'INDUSTRIAL' crops (not shown). Here 'Forage Plants' are defined as bulk fodder crops such as vegetative legumes and grasses, fodder maize, and fodder roots including forage beet (*Beta vulgaris* spp.).

In addition to both UK and EU policy, there has also been huge land use change globally involving the destruction of forest land to produce agricultural land [842-844], with a 3 % expansion of world pastures and croplands from 1985 to 2005 [845]. This land use change increases soil erosion and loss of nutrients decreases biodiversity indexes and is particularly evident in the tropics where deforestation has ravaged rainforests [846; 847]. However as can be seen in Figure E5, FAO data of the last few decades show an increase in forest areas in Western European countries [843]. This includes the UK, and its dried forage competitors of France and the Netherlands. A new analysis of satellite data also suggests that tree canopy cover has increased across the globe between 1982 - 2016, although land degradation to bare ground is also apparent [848].

However, the suggested increase in forest cover, at least for Western European countries, is because the conversion to agricultural land happened pre-1982, by preindustrial communities [849]. Thus, small increases in recent years look impressive but not when compared to the original landscapes of each country. Therefore, degradation of land has been long underway in the UK, with serious negative consequences and some subsequent effects for farming and ecology are discussed below. This means it is imperative the UK government take seriously Afforestation policy and does not only apply regulations to tropical countries where deforestation is a recent practice, but also temperate climates. This includes reacting to guidelines set by the EC for the green direct payment scheme in relation to Ecological Focus Areas (EFA) on arable land [850], and also continuing to improve the current governments Greening Policy [851].

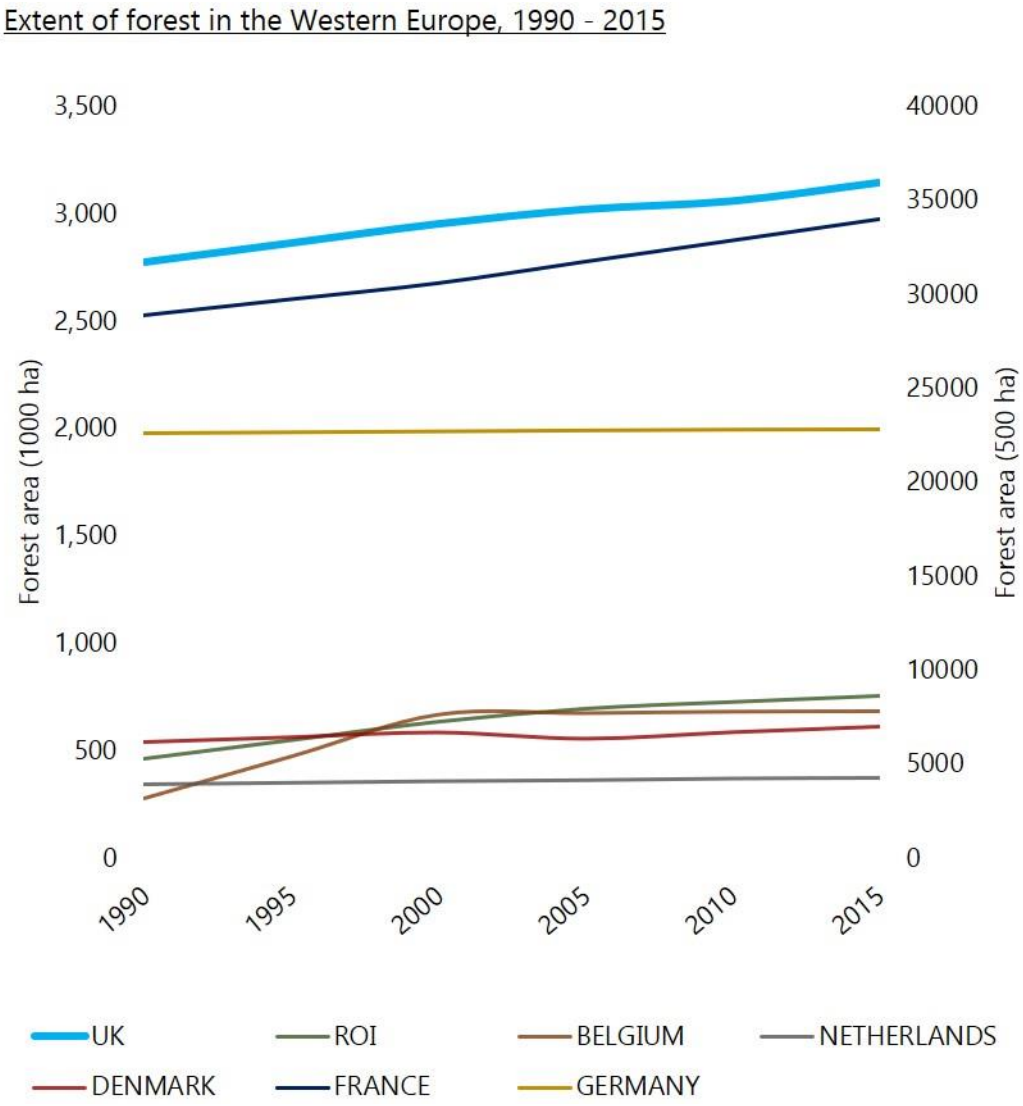


Figure E5: Extent of forest of the UK and surrounding Western European Countries from 1990 - 2015, data from from the "The Global Forest Resources Assessment 2015: Desk reference" [843].

Note beforehand for ease of design both France and Germany are read using the right axis (500 ha) and the rest are read using the left axis (1000 ha) In the original report this data is in "Table E2. Extent of forest 1990-2015", found on pp. 9-14, and is under copyrighted © FAO, 2015

E.3 POLICY IMPLICATIONS OF NITROGEN FERTILISER USE IN DRIED FORAGE PRODUCTION

As touched upon in the above section, a large amount of legislation changes for the forage crop industry have been in restrictions on N fertiliser usage. Nitrogen has long been known to be fundamentally important for plant growth. Deficiency seriously limits crop production through restricting protein assembly, affecting both primary and secondary metabolic pathways. This is particularly true for the high N demand of the leaf photosynthetic apparatus where deficiency impacts on all growth parameters [75]. Leafy crops like vegetables, grasses and leguminous herbs have a slightly larger N content than cereal grain crops and therefore require more N for optimal production [74]. These crops include those grasses and legumes grown for high temperature drying such as those produced by the BAGCD for high N protein concentration forage in Table E1.

The development of Haber-Bosch process for fixing free nitrogen (N_2) from the air and converting it into ammonium (NH_4^+) is arguably the most dramatic event in modern human history. This fixation process was part of the Green Revolution that allowed crop production outputs to increase four-fold [421]. Fritz Haber and Carl Bosch's process was developed from Haber's 1908 "synthesis of ammonia from its elements" patent [423]. The work won each of them a Nobel Prize. More notable than its scientific advance of chemistry, ammonium fertilizer production changed the impact of the late-industrial age globally by feeding 50 % of the world population through both increased grain yields, but by also improved forage crop yields which in turn supported a growing animal production industry [424]. This population boom is shown in Figure E6, adapted from UN statistics from 1700 to 2015 [852; 853]. Nowadays, anthropogenic activities fix 210 Tg of N annually [427], a figure set to continue for the foreseeable future [440], with total N input for food production globally at 171 Tg N $year^{-1}$, half of which is accounted for by the animal products industry [428].

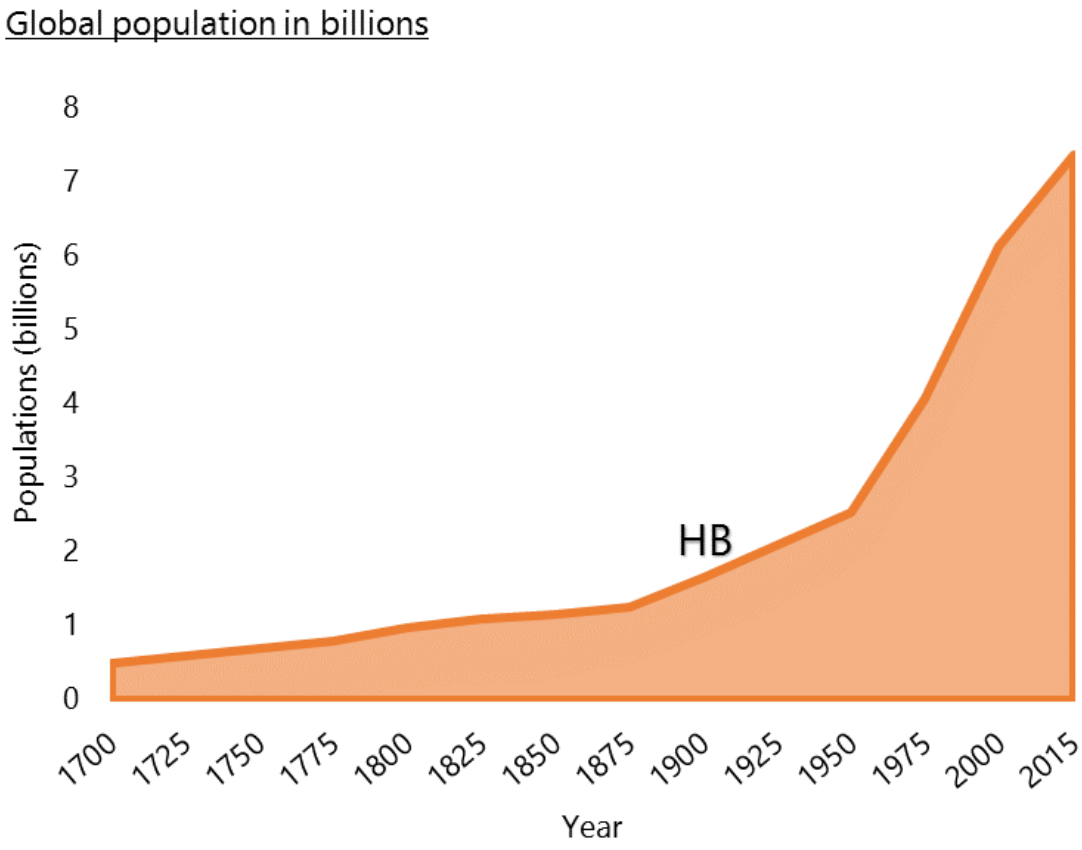


Figure E6: Global population since 1700 to the present day; adapted from "World population to 2300" and "World Populations Prospects: The 2017 Revision" [852; 853].

'HB' is the development of the Haber-Bosch process in 1908.

There are some momentous problems with N fertiliser use. The process is resource and financially exhaustive; the practice requires huge quantities of energy that for the UK is mostly generated through reserves of natural gas [854]. Global N demand is also being stretched by its potential use in transport fuel and space heating with further technological advances on the process multiplying [436]. Emissions of N from fertiliser is estimated to be at around 10 % [437] with the bulk of these caused by agricultural emissions from animals and manure [452; 438]. Previously, emissions have been shown to be highest in Asia, predominantly China and India, and Europe [439]. However, other areas of the world are now catching up due to further increases in synthetic fertilizer use intensity [393]. As of 2015 Western Europe used 7.3 % of the world's N consumption [440], disproportionate with its landmass size; the growth in N fertiliser use has decreased by almost 1 % for the area, however consumption still stands at a forecasted 7.13 % [449].

Due to the importance of ensuring N fertiliser is applied responsibly, a lot of policy has been introduced both through the EC and UK government. Arguably, the most significant of these for the dried forage industry has been implementation of the Fertiliser Manual RB209 8th addition [79]. The publication set-out stringent acceptable levels of N fertiliser use across growing seasons built on several policies; the 1991 Nitrate Directive to protect N fertiliser from agriculture polluting ground and surface waters [76], the 1998 Action Programme for Nitrate Vulnerable Zones covering England and Wales [77], and the subsequent 2008 Nitrate Pollution Prevention Regulations for England [78]. Amended annually, this guide provides guidelines for fertiliser management of each specific crop type, including grasses and forage crops. As a document it was originally very strict about the amount of knowledge farmers were expected to have about N use, and desired in-depth testing of N soil content through season, and proof of application needs. The RB209 did promote many sustainability practices by offering growers the opportunity to gain Environmental Stewardship (ES) points that could be converted into funding if they conform to the rules [79].

In both the RB209, and the 2008 Implementation of Nitrates Directive in England report that initiated many of its guidelines, the recommended maximum N application rate for dried forage growers was defined as 370 kg ha^{-1} over a growing season [412; 79]. This figure uses both the initial application rates for grassland, particularly for dairy-grazed grasslands, and the extra allowance of 40 kg ha^{-1} permitted after cuts [79]. Based on DEFRA statistics this would be enough to achieve a yield of 11.3 tonnes DM ha^{-1}, however this estimate did not consider the level of cutting the dried forage undergoes. The intensive cutting anywhere from 4 – 7 times between spring and autumn means that the forage crop, whether grass or legume, is subjected to high N demands. Especially affected is the chlorophyll content, which is an important characteristic of dried forage appearance as well as a by-product extracted for other industries. This means if BAGCD members stuck to the guidelines they would not reach their usual yields and production of the dried fodder would be lower than the CAP presumed, thus not increasing their single farm payment until years later.

Therefore, the BAGCD commissioned its own report to appeal to DEFRA to reconsider its guidelines stating that the N max "limit was developed for a different grass production system in mind (i.e. grass production on dairy farms)" and therefore is not in-line with dried forage needs [486]. The request was accepted and included in subsequent legislation from 2013 onwards [414], as detailed below in Figure E7:

"Grass grown for dehydration or chlorophyll production: You can use nitrogen up to the level recommended in writing by a FACTS-qualified adviser if you're growing grass to achieve a protein content of at least 16% in the dried product. If the land is irrigated, you must not use more than 700 kg of nitrogen per hectare. If the land isn't irrigated, you must not use more than 500 kg of nitrogen per hectare."

Crown© (2013). "Guidance on complying with the rules for Nitrate Vulnerable Zones in England for 2013 to 2016 ", (ed.) Defra. (London, UK).

<u>Figure E7:</u> The vital exemption for BAGCD members in the RB209, outlined by the Crown© [414].

This exemption means yields by dried forage growers can be maintained despite huge nitrogen requirements for the crop during the growing and cutting season.

Such high guideline usage does mean UK dried forage growers must show evidence routinely to DEFRA and, as mentioned, be signed off by a FACT-qualified adviser (a body offering training for agricultural guidelines run by BASIS (registered charity No. 1077006)). The only other strict requirement of the RB209 currently is that all farmers must stick to the Code of Good Agricultural Practice (the CoGAP), which sets out how to limit nitrate leaching and must be followed to gain the basic payment scheme of the CAP [413]. In addition to abiding these guidelines, BAGCD also set up their own trials to optimise and hopefully limit N fertiliser use, as this is also of excessive cost to the grower. However due to their high N max limit, the dried forage producers tend to be left out of annual reporting as an exempt group [442]. The average application of fertilisers for English farms is currently 113 kg N ha^{-1} (along with 19 kg phosphate ha^{-1} and 26 kg potash ha^{-1}) [415], whereas BAGCD members can apply up to 700 kg N ha^{-1} [83].

E.4 IMPORTANT RESEARCH DEVELOPMENTS FOR THE INDUSTRY

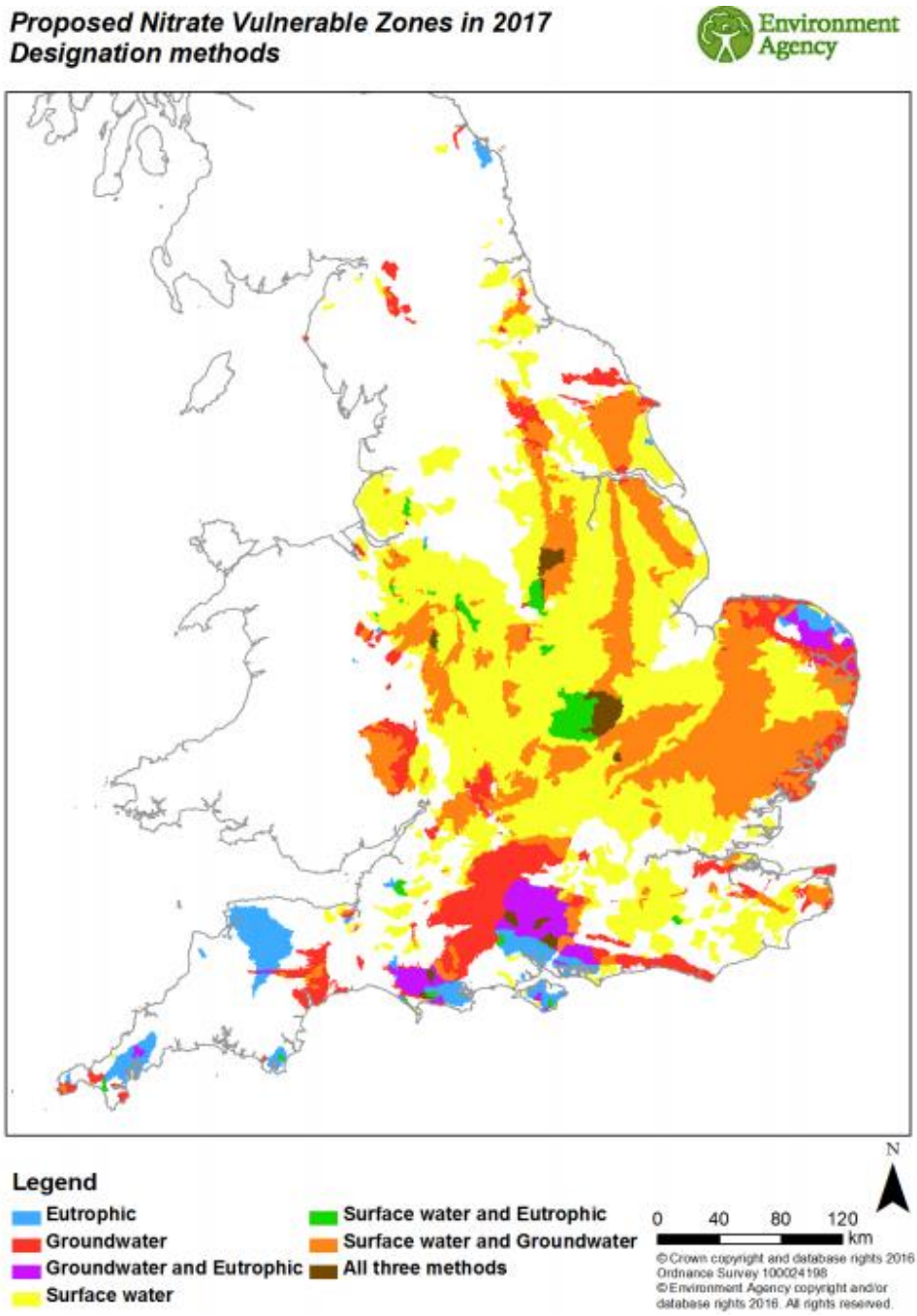
The above policy changes have been largely affected by scientific developments in many fields related to agriculture, from ecology and conservation biology to plant cell biology and genetics. Three of the principal areas where research has affected the UK dried forage production industry are discussed below; Nitrogen Fertiliser, Leaching and Emissions; 'Soil Health' and Forage Crop Cultivation; and Biodiversity and Greening Policies.

E.4.1 Nitrogen fertiliser, leaching and emissions

As extensively discussed in the previous section the use of N fertilisers is of imperative concern to the UK government. Fertiliser resource consumption and subsequent emissions from their uses have dramatic consequences for the world. The vast energy required puts a strain on our current energy demands and using a huge supply of natural gas directly contributes to our unsustainable dependence on fossil fuels. This directly

influences governmental policies and is constantly monitored in developed countries [440; 449; 450] and in the UK specifically [442]. There is increasing emphasis to ensure the N component of manure is adequately exploited, especially for degraded soils [450]. Increased global demand for protein estimates of 110 % ± 7 % [463] will undoubtedly exacerbate the current problems with N fertilisers as an increase in food production is required. Due to this, there are community, political and financial aspects to using less synthetic fertilizer overall and manage those used more efficiently. Interest in N fertiliser use include the topics of both the leaching and gaseous emissions from N fertiliser use. Below N leaching and emissions are discussed separately.

Leaching of N leads to eutrophication of water supplies where algal blooms in rivers and lakes limits sunlight, space and oxygen for aquatic species and therefore leads to high death rates in these ecosystems. Such leaching can also contaminate human drinking water especially in ground water supplies. Forage grass and legume crops have long been known to have high rates of leaching to the environment [413]. This problem can be exacerbated when they are grown in sandy soils, the choice for many forage crops. Some estimates calculated that 60 % of applied N is lost through leaching, run-off, denitrification and consumption by microbial populations [476; 477]. Furthermore, areas termed Nitrate Vulnerable Zones (NVZs) make up approximately 58 % of the land in England, with an increase in total area of 1300 km^2 since 2013 [81; 82]. NVZs are determined by land gradients, ground cover (especially fallow or uncultivated land), water sources, soil types and erosion intensity, and weather conditions [83]. Figure E8 shows the most recent mapping of NVZ zones across the UK [82], with many of these in BAGCD farming areas. Not only are there arguments for limiting N leaching due to effects on soil degradation, soil pH and biodiversity (terrestrial and aquatic shown in Figure E8), but also for the impact of drinking-water containing leached N on human health, resulting in decreased life expectancy [446-448]. This has driven assessment studies into the social cost of nitrogen (SCN) [445].



<u>Figure E8:</u> Nitrate vulnerable zones (NVZs) map of the UK for 2017, directly reproduced from the "Review of Nitrate Vulnerable Zone designations for implementation in 2017: Environment Agency report and recommendations to DEFRA: Report Number HOEV151604/R" [82].

In the original report this map is "Figure 4.2 - Proposed 2017 NVZ and the methods that designate each area", found on pp. 34, and is under copyright: ©Crown copyright and database rights 2016 Ordnance Survey 100024198 and ©Environment Agency copyright and/or database rights 2016.

Research into fertiliser effects on grass has long been carried out including long-term experiments such as Rothamsted's Park Grass Continuous Hay Experiment, which began in 1856 [479; 104; 480]. This included a 19-year assessment of leaching using ^{15}N labelled fertiliser, finding leaching of $^{15}NH_4$ and $^{15}NO_3$ to be approximately 13.9 % and 21.9 % of total N application, respectively [481]. Many forage grasses, such as ryegrass, will preferentially uptake nitrate (NO_3^-) as N source particularly at high fertiliser rates [469; 470]. However, as a grower may choose to cultivate mixed crops of grasses and/or legumes this preference may vary. For a long time, studies have also looked at the direct influence of N sources on final product of dried forage yield, from its early industry [63] to those carried out now on many of the BAGCD current sites. Studies have shown that the leaching of inorganic N is lower for grassland than that of arable land [483; 484]. This is largely due to the N applied being routinely removed as the plant uptakes the required amount, and then furthered removed by the plants after cutting, as discussed previously [485; 486]. However, as dried forage cropping is often seeded each year in the UK, and is not a permanent pasture, it may have a leaching potential more like arable land in discrete periods.

There are strong suggestions that N emissions may contribute to climate change, with nitrogen oxides (N_2O and NO) being the single largest contributor to atmospheric greenhouse gases [459]. Such emissions can be produced as a by-product of N fertiliser use through microbial breakdown, both on farmland (direct) or off farmland due to leaching (indirect). Figure E9 shows the trend of UK nitrogen oxides emissions along with ammonia (NH_3) and non-methane volatile organic compounds [855]. Although such emissions have been decreasing, the majority of emissions are of agricultural origin through N fertilisers, at around 75 % [456]. In many other countries emission quantities are not decreasing.

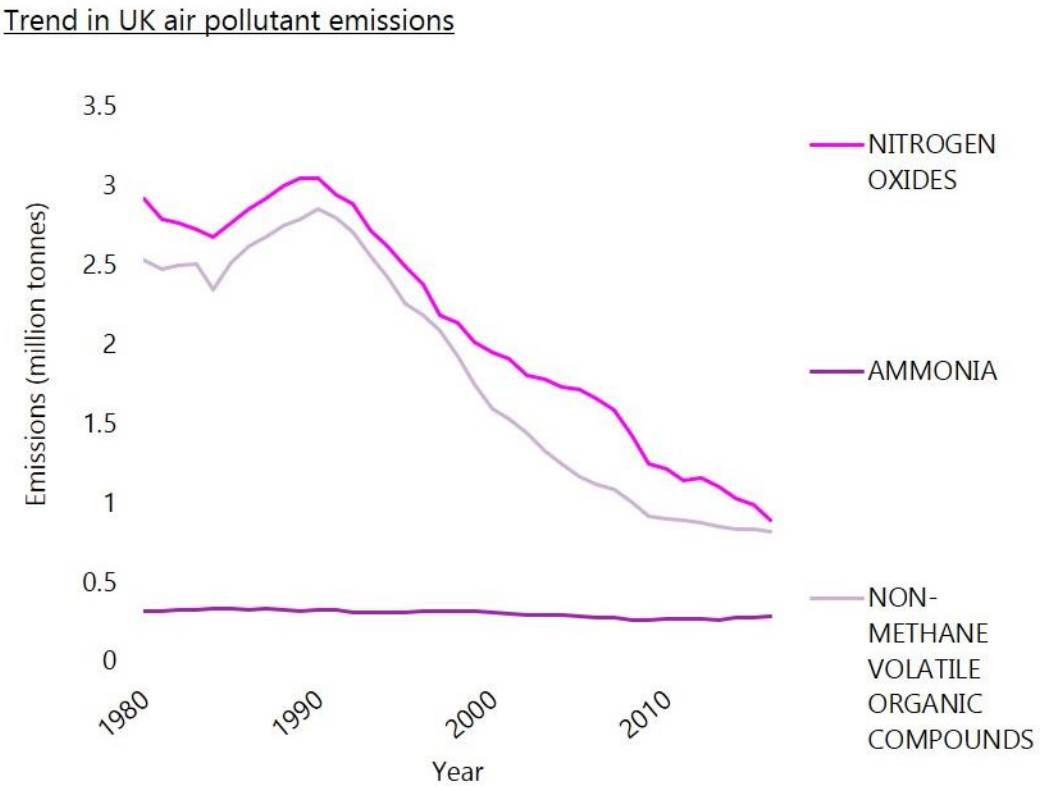


Figure E9: Trend in UK air pollutant emissions in million tonnes including Nitrogen Oxides (NO and NO_2), Ammonia (NH_3) and Non-Methane Volatile Organic Compounds (NMVOCs).

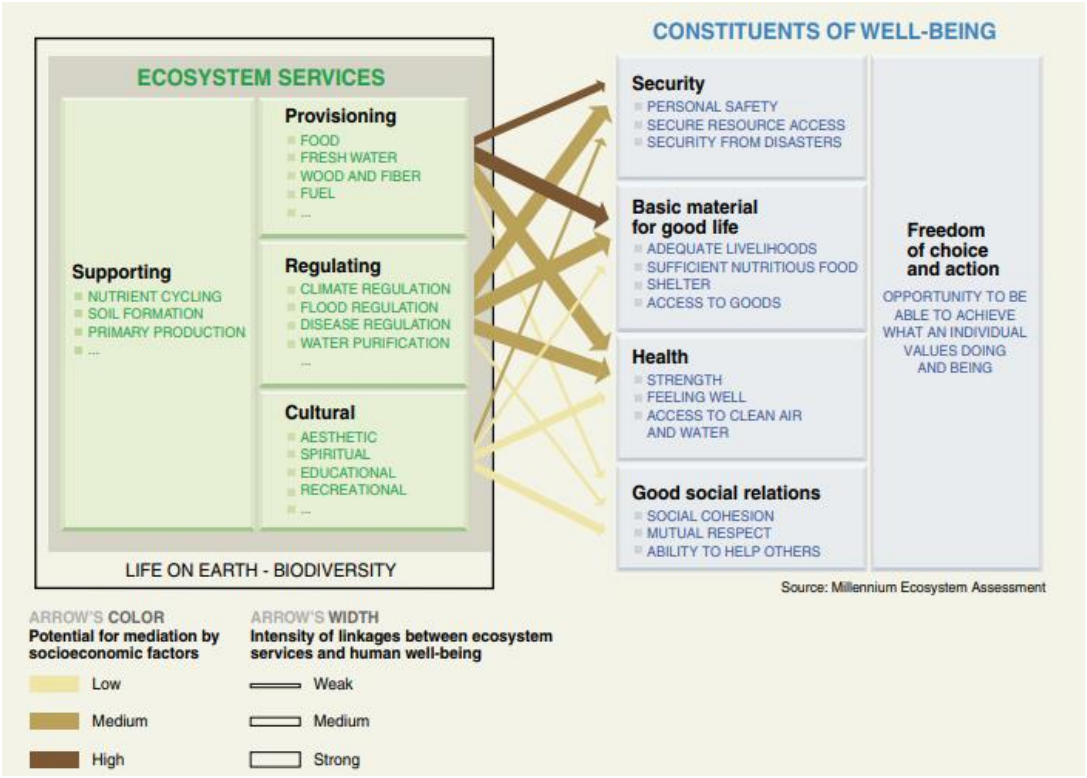
Data is from DEFRA, and is for the years between 1980 and 2016 [855].

Emissions are not just related to the quantity of fertiliser applied, but also the type of fertiliser and the crop [113; 114]. At present there is limited data for grassland emissions grown specifically for cutting and drying [115; 116], but it can be presumed to be similar to the typical agricultural emission amounts in order to adequately reduce UK greenhouse gas contributions. Care should be taken to ensure that trying to reduce emissions does not reduce the efficiency of production. For example, through simulations it was found that reducing N_2O emissions by ~ 4 % would require a N fertiliser application reduction of ~ 10 % which would decrease yield ~ 2 % [856]. This means that although the policy to decrease emissions may be successful, such as those outlined in various reports [857-859; 557], it may be detrimental to the intensive agriculture system, thus less fertiliser may be applied to the land, but more land is utilised to grow a similar amount of crop. Practices such as low or zero tillage may be able to aid with this emissions problem [582; 88; 90; 92], but more crop and cultivation specific evidence is required. Many countries are signatories of the Kyoto agreement [860], which details the need to decrease emissions. However, care must be taken on a country to county basis, so that adhering to N fertiliser limits does not decrease the efficiency of the system. It is therefore necessary to have management-based studies into N application rates with parallel, accurate quantification of emissions for each arable land type, including forage crops grown for drying [861; 113].

E.4.2 'Soil health' and forage crop cultivation

Across Europe between 1700 and 1950 around 70 % of the original forests and wild grasslands were converted to cropland, including forage cropland [101]. With these changes came new cultivation problems as soils become more vulnerable to erosion and nutrient losses. The status of UK agricultural soils is quickly moving up the list as successive governments are prioritising 'ecosystem services' [413]. Ecosystem services have a long history in both ecological economics and agricultural sciences [862] with the term first coined in 1981 after many decades of development [863;

864]. Figure E9 is taken directly from the Millennium Ecosystem Assessment, and explains interactions between all types of ecosystem services, as well as how they impact on the well-being of a community [101]. By looking at Figure E10 one can see that the soil is a crucial service provider which can be separated into four major categories; supporting, provisioning, regulating, and culturally. Supporting through its structure and formation, provisioning through plant growth, regulating especially in flood and erosion defence, and culturally as aesthetic landscape topology is important for recreational and spiritual practices. Another term used increasingly in UK policy and economics is 'natural capital' [93; 94] and this summates the ecosystem services such as soil, with addition of the available stocks or biodiversity of an area. Consequently, the UK government has appointed the independent advisory Natural Capital Committee (NCC) [865], which works in collaboration with the international Natural Capital Coalition organisation, which also includes the United Nations and EC, as well as discrete representatives of America, Switzerland, the Netherlands, China [866].



<u>Figure E10:</u> 'Ecosystem services' and their link to 'Constituents of Well-being', directly reproduced from the "Ecosystems and Human Well-being: Synthesis" [101].

In the original report this diagram is "Figure A. Linkages between Ecosystem Services and Human Well-being", found on pp. vi, and is Copyright © 2005 World Resources Institute. All rights reserved under International and Pan-American Copyright Conventions. No part of this book may be reproduced in any form or by any means without permission in writing from the copyright holder: World Resources Institute, 10 G Street NE, Suite 800, Washington, DC 20002.

'Soil health' is a limited term to denote a soil system providing optimal ecosystem services through its biological, chemical, and physical characteristics. It has been extensively reviewed in terms of universal soil quality factors [867], those specifically assigned to agricultural systems [868] and for parameters of sustainability testing [869]. The main areas of soil health that are threatened in the UK are the decline of soil carbon in arable and peat soils, soil degradation (especially for peatland with subsequent carbon emissions), contamination of land with heavy metals, and a lack of soil monitoring schemes [397]. Specific reviews for both Wales and Scotland [870; 395] are available, as well as EU directives [871]. It is felt by the current UK government that the CAP encouraged farming that led to these problems through intensive agriculture, which has depleted nutrient and humus levels, and eroded or compacted soils [93]. As with all UK agriculture, dried forage growers are facing the same soil health threats. Two of the main UK soil health research questions at present are how to decrease soil erosion levels in arable and grassland, and how to increase the soil organic matter (SOM) of these depleted areas [405].

Through meta-analysis it has been shown that the erosion rate of agricultural land and orchards are similar to that of bare land, approximately between 1000 – 10000 mg/km^2 for their highest probability density whereas areas such as forests and shrubland are lower [551]. This is partly due to a lack of established plants; arable agricultural systems lack the development of complex root architecture under most intensive management practices. Permanent grassland is better able to prevent erosion, as well as sequestering 34 % of global carbon stocks due to their stability [86]. An in-depth review has demonstrated the importance of vegetative cover for controlling, limiting or in some cases, reducing soil erosion [872]. The cost in £ ha^{-1} of productive land loss through rotation changes or becoming a non-productive area due to soil erosion has been calculated for many UK crops, showing how broad the problem is across farming [552].

As with N fertiliser use, forage crops used for drying are highly cultivated and should therefore be managed like other intensively farmed land until more evidence is found to suggest otherwise. However, promising work on the use of grass and/or legume mixes to reduce soil erosion is being established, which is particularly relevant for forage growers who already trial such mixes. As soil erosion is linked to a more complex root architecture for many plant species, work has shown that crops such as ryegrass (*Lolium perenne*), are particularly good at reducing erosion due to their fibrous rooting pattern [110]. BAGCD crops (Table E1 and E2), of Lucerne (*Medicago sativa*), and red clover (*Trifolium pratense*), are also associated with increased soil aggregate stability when continuously cultivated and could be promising for combating erosion [111].

Depletion of SOM is not only exacerbated by soil erosion, but has a direct impact on future crop yields [873]. This could mean that the breeding of crops with improved root architecture could not only improve their erosion limiting potential, but also increase SOM [874]. Long-term testing of soil health for SOM is also complicated by the impact of climate change [875]. Studies have shown that use of lucerne-grass mix can increase SOM, especially when coupled with lower tillage [876]. As seen with grass and clover leys, which have long been known to improve erosion problems and increase SOM [112]. For forage crops to become useful for limiting these soil health problems, it is important they are cultivated with a long establishment period, using minimal tillage [553]. For these reasons the use of appropriate minimum tillage and the reintroduction of grass buffer zones are part of many governments' policy, including the UK [93]. Buffer zones are also being introduced to aid biodiversity levels, which will be discussed in the next section.

E.4.3 Biodiversity and greening policies

As with the sections above, N fertiliser leaching and emissions in conjunction with soil health problems have a negative impact on biodiversity, which is also declining due to conversion of the land for different uses. These factors have been observed to effect biodiversity

globally [844]. Although the conversion of natural UK woodland to agricultural cropland was prehistoric and preindustrial as discussed above, the negative consequences of land use change are current. This is especially true when coupled with more recent changes to UK agriculture including the abandonment of mixed farming systems in favour of intensive cropping, grazing regimes, and pesticide/fertiliser usage. The causes of UK biodiversity decline in cropland include many drivers explained in Burkmar and Bell's 'Drivers of Biodiversity Loss' report for the Field Studies Council. These can be listed as habitat loss, destruction and fragmentation; pollution from farming practices including N fertiliser usage; biotic exchange of invasive species through agricultural trade; and climate change influence especially regarding GHG emissions [877]. In the collaborative State of Nature 2016 publication it was reported biodiversity is declining in the UK from 1970 to the present day in all land use areas, apart from debatably in urban and total marine (excluding fish stocks) populations [878]. It is likely the decline in biodiversity in non-agricultural areas is still broadly affected by the same drivers as cropland, including agriculture's role in climate change.

Semi-natural, managed, temperate grasslands have been designated as very significant examples of rapid decline of biodiversity due to pollution from N fertilisers (and phosphorus; not discussed in this report), and significant habitat changes [101]. It has been shown that the application of N fertiliser on UK managed grassland can decrease diversity in both short- and long-term experiments for animal, plant and microbial species [102-109]. There are some disagreements whether the form of N fertiliser being used varies this decrease [879; 880]. Declines or changes in microbial species diversity have also been linked to declines in human health [881]. Biodiversity is not just important for mitigating declines and changes in individual populations, it also provides crucial information to quantify other ecosystem services in an environment [882; 590]. Thus, one can determine problems in other areas, such as soil pH or long-term decreased flood defence, are dependent on species population variations. Such insight has led the NCC to increase its push to quantify natural capital as a complex entity, with biodiversity interlinked to ecosystem services [883; 884].

However, it is important to recognise that pushes to increase biodiversity numbers may be inherently flawed for agricultural systems. By increasing biodiversity through many initiatives, you are likely to decrease system efficiencies, which could mean that more land would be required for agriculture to produce the same crop yields giving a cyclical feedback that reduces biodiversity. Research is needed to find a compromise between system efficiency and biodiversity directives; the balance between these two systems is a concept termed 'ecoagriculture' [587]. Permanent grasslands are capable of increasing biodiversity-richness and genetic variability, including traditionally managed European mixed grasslands [86], which are similar in composition to the forage cropping of BAGCD although less intensive in terms of tillage and rotations. For example, a huge driver of biodiversity decline is the wide use of herbicides for unwanted weeds, so management systems that lower weed populations but do not affect non-targeted, wider biodiversity is required. Approaches that naturally suppress weed population such as low tillage [885], or studies of productive grass and/or legume seed mixes with competitive root architecture [886; 887], are promising for forage growers. Moreover, grassland has high rates of biodiversity stabilisation after ceasing long-term N fertiliser use [888], and so current forage cropland may be fast recovering.

UK agriculture must comply to many EU rules including the Birds and Habitats Directives and Natura 2000 Network, detailed as part of the EU LIFE programme [589]. The EU has also been imperative in allocating funds to 800 LIFE-Nature Projects, with a total of € 1.3 billion between 1992 – 2005. This funding aim to maintain and restore natural habitats whilst working with both conservation and farming groups. The UK government also has a long-term commitment of funding biodiversity projects, as shown in Figure E11. However, Figure E11 also reveals since 2008 the % of GDP spent on biodiversity projects has decreased, reflecting a change in government priorities due to the transition from a Labour to Conservative cabinet.

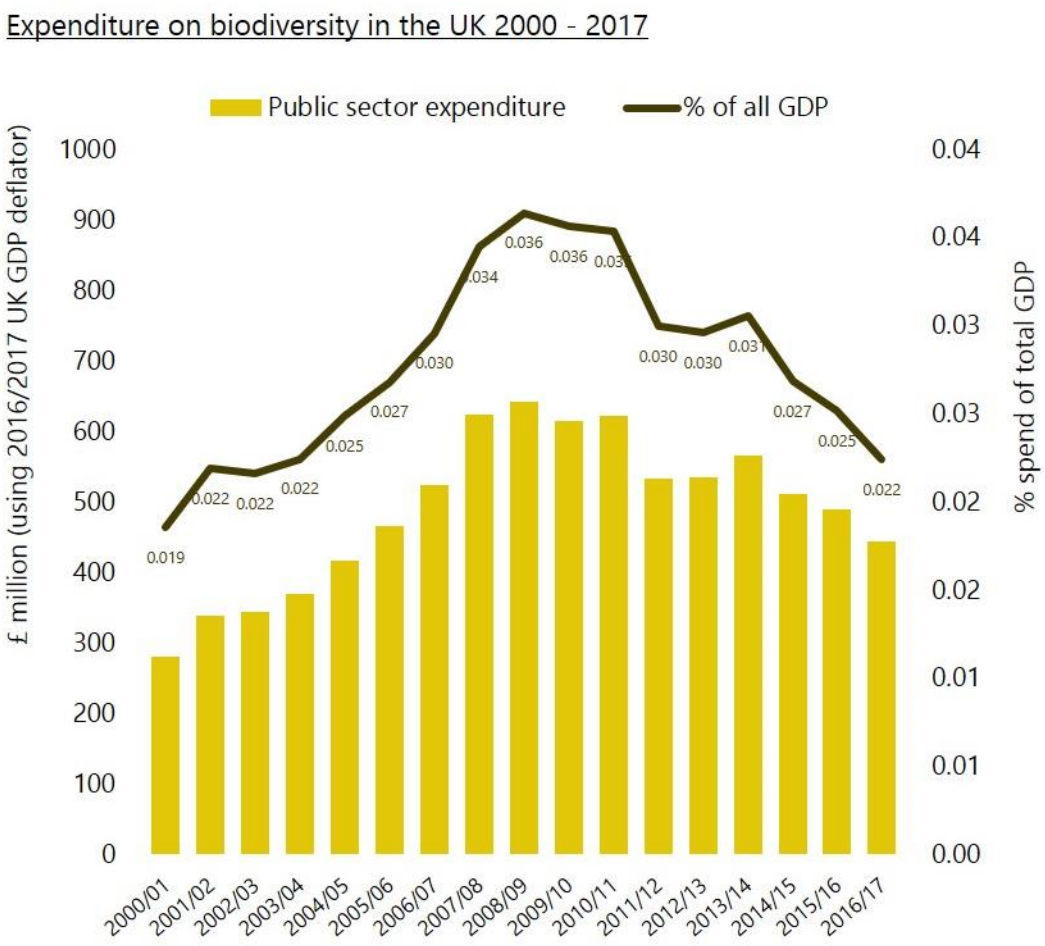


Figure E11: 'Expenditure of UK biodiversity', data from the "UK Biodiversity Indicators 2018" [889].

In the original report this diagram is "Figure E2i. Expenditure on biodiversity in the UK, 2000/01 to 2016/17.", however here NGO data have not been included, and is Copyright © Crown 2018.

E.5 THE FUTURE, INCLUDING POTENTIAL POST-EU EXIT CHANGES

This report has discussed the past production of UK forage cropping for dried feed, and the present problems that the agricultural industry face at large, especially in terms of policy restrictions. The future of UK agriculture is currently uncertain, especially with its exit from the EU (Brexit). Such a change will affect the policies made by future governments, which are unknown.

The EU single market means that not only is agriculture heavily subsidised by the CAP, but it is also protected from outside markets [586] through the Common Market Organisation (CMO). The CMO does this through intervention at problematic agricultural times, marketing of products especially in terms of safety, and managing both internal competition rules but also international licenses, tariffs and processing of products [890; 891]. This means there are already strong focuses on protecting farming livelihoods especially through the EC's Agricultural Markets Task Force. This organisation has been imperative to the recent proposed legislation on protecting the business rights of farmers, but also with specific objectives to "maintain market stability, enhance agricultural producers' income, [and] improve agricultural competitiveness" [892]. The chance of improving UK agricultural profits may be lost through the Brexit movement. Critical issues such as the protection of farmer business and trading rights maybe overlooked compared to all the other political objectives the UK government deems important.

The ecosystem benefits of restoring grassland globally are well documented [101]. Even when a mixed forage cropland is cultivated rather than permanent grassland, there are benefits and it is still preferential to the cultivated cereal monocrops. Mixed forage crops can bring improvements in soil health and biodiversity as discussed above whilst still retaining their commercial value. The main problem with moving away from monocropping is the reliance of the livestock industry on the use of concentrate feeds, such as cereal grain and biproducts, rather than forage crop vegetative biomass. Concentrates deliver high nutritional value

especially in crucial developmental animal stages (immature livestock, pregnancy, laying, milking, etc.), but come with a higher land use affect because they compete for land area that is also used for to produce cereal grain for human consumption. Forage crops however, can be grown on lower grade land not suitable for cereal production. The current global feedstock rations provided to livestock are shown in Figure E12 below, with fodder crops having a small portion of inputs [893]. Therefore, if more intensive cereal cropland was transformed to mixed forage production, to provide a similar level of animal products more land would be required. A solution may come from a switch by many western populations to more plant-based diets. Such a lifestyle change could conceive a lower reliance on cereal concentrates, enabling livestock to still be integral to diet and culture, but produce increased positive impacts for ecosystem services. However, the problem with such an approach is the lack of knowledge of how much cultivated grassland equates to ecosystem service provision of permanent grassland.

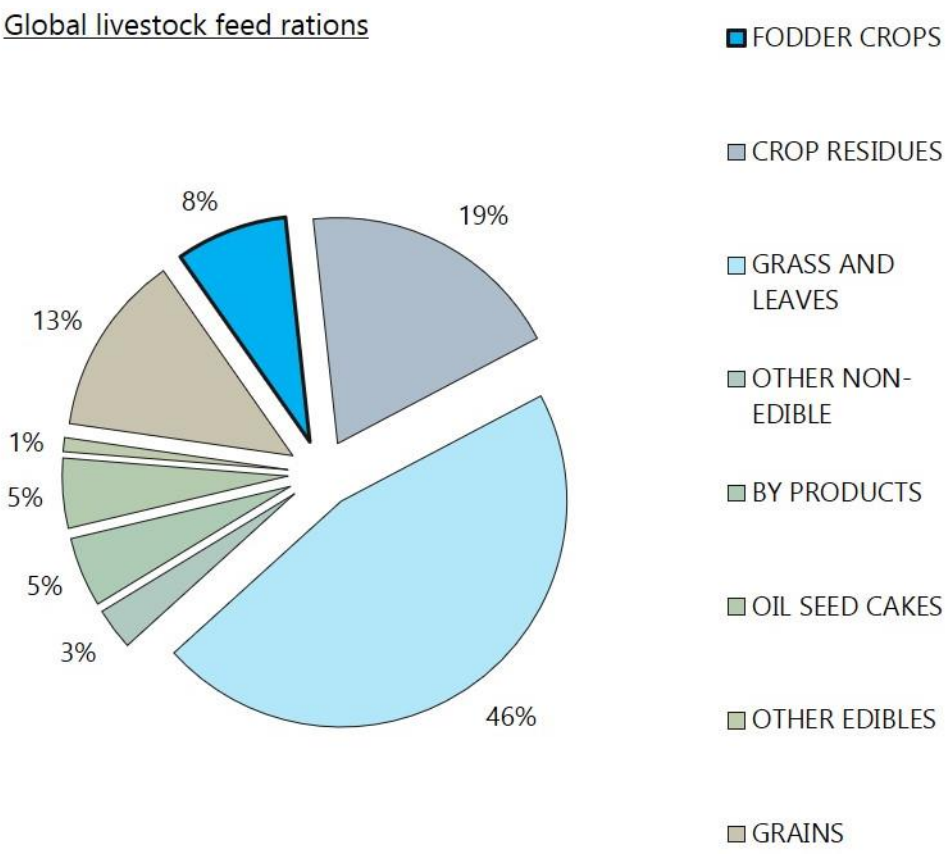


Figure E12: Global livestock feed rations, adapted from "GLEAM 2.0 - Assessment of greenhouse gas emissions and mitigation potential" [893].

In the original report this diagram is "Global livestock feed intake" and is Copyright © FAO 2018.

Of prominent importance for the future is not only the N emissions discussed above, but also total GHG emissions from agriculture and forestry of which net carbon losses globally where they contribute to 24 % of total output [894]. These GHG emissions include CO_2. This is important as although CO_2 has a lower 'warming' potential than other GHGs, such as nitrogen oxides (NO, N_2O), it is more publicly recognised due to a longer history of journalistic coverage. This means general industry policies on total GHGs, and not just N emissions, are huge political drivers, and thus may continue being the focus for successive UK governments rather than more detailed policies for specific agricultural land-uses including forage cropland. Governments need to understand that a 'one size fits all' policy cannot be applied to agricultural policy.

Although the challenges of curbing N fertilisers use, increasing soil health, and decreasing the negative effects of soil erosion and biodiversity should be high on the UK government's agenda, it is important that numerous policies are not at the detriment to farmer's livelihoods. Although policies such as the Greening initiative should be continuously improved, it is imperative that government also continue to fund agricultural science to explore these complex interactions. Agriculture is heavily restricted and regulated, which should continue to improve the wider community, but care should be taken to ensure farming is still appreciated and not low on priority lists.

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