Improving crop phosphorus uptake through use of bioinoculants

A thesis submitted to Bangor University

by

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of

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Summary

The soil microbiome contains fungi which deliver key functions in all ecosystems. In agricultural contexts, a number of benefits relating to crop yield, health and morphology are directly derived from soil fungi. This has in recent years resulted in the commercial utilisation of such fungi as biostimulants, which are purported to deliver crop benefits. A key difficulty remains that efficacy and functionality of such products remains very difficult to quantify, and where beneficial effects have been observed, the modes of action responsible remain unknown. Much remains unknown concerning interactive effects between microbes, soil and crops. There is a tendency for existing studies to depend on a small number of growth indicators (chiefly yield), and in this thesis, a case is presented that future research should consider a greater number of morphometric variables and plant health indicators in plants treated with biostimulants in order to reveal more useful insights into the effects and efficacy of these products. In this study, biostimulant treatments applied to an experimental *Lolium perenne* crop were associated with significant increases in plant area, perimeter, height and biomass, and were also linked to improved crop phosphorus uptake in the leaves and roots. The most effective biostimulants studied consisted of mycorrhizal fungi and plant growth-promoting bacterial formulations biofixed to diammonium phosphate and zeolite carriers. These these growth benefits were largely attributable to mineral effects as opposed to the live component, but the results suggested a possible persistence effect of improved plant growth-promotion, since similar yields were obtained over the course of three non-destructive cuts undertaken over a three month period. Similar treatments on an *Arabidopsis thaliana* experimental crop were associated with similar morphometric results, but this time the crop P uptake was lower in biostimulant treated crops than in the controls, suggesting a possible metabolic cost to the plant associated with the treatment. Finally, a high-throughput DNA metabarcoding method was used to examine fungal community structure shifts associated with sustainable intensification approaches in a field trial to contextualise the findings of the previous chapters, and examine the effects on soil fungal communities of representative management approaches anticipated in the future that will involve biostimulants. The approach was also able to demonstrate divergent microbial communities, increased AMF abundance with sustainable intensification approaches and an increase in the relative abundance of economically important plant pathogens. Overall, while potential exists for biostimulants in agriculture, much work is necessary to improve consistency, viability and the knowledge base before they can be used with confidence.

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Chapter 1

Introduction

1. General Introduction

This study was funded by a Knowledge Economy Skills Scholarship II (KESSII). KESSII is a major European convergence programme, led by Bangor University and benefiting from industry support to conduct postgraduate research. Industry support was provided by the Glenside Group (Glenside Group, 2020). Glenside produce fertilisers, biotsimulants and run soil tests for arable and livestock farmers which are designed to improve resource use efficiency and productivity.

The overarching aim of the work presented in this thesis was to examine the use of biostimulants in agriculture, and to explore the mechanisms through which microorganisms can increase crop yields, with a particular focus on the impacts on plant availability of the macronutrient phosphorus (P).

1.1. Research rationale

Global use of chemical fertilisers in agriculture has in recent decades shown significant increases and has corollary implications in the form of soil, air and water pollution (Vassilev, 2015). This trend is occurring in response to increasing food demands from a growing world population. Methods of food production must expand sustainably in order to meet the needs of the world's food demands, in balance with a stable environment (FAO, 2009). A major concern for agriculture is the supply of phosphorus, which is a vital mineral for plant metabolism (Campos *et al*., 2018). Mineral phosphorus used in agriculture is finite and non-renewable, as well as volatile for dissolving into solution with rainfall, and contributing to eutrophication (Richardson, 2001).

A possible component of an integrated solution to this problem is the utilisation of plant growth-promoting fungi as biotimulants in agriculture, which has attracted increasing research interest (Rouphael *et al*., 2015). Some fungal taxa have been demonstrated in laboratory and field experiments to be significantly associated with improved crop P uptake (Detheridge *et al*., 2016), and as such, may offer the potential to reduce levels of inorganic fertilisers inputs for crop production (Owen *et al*., 2015). A number of plant growth-promoting fungi have been shown to mobilise pools of phosphorus which are not directly available to plants (Ruzzi *et al*., 2016). In agricultural contexts, previous P amendments from fertilisers and manures has often resulted in relatively high 'legacy P' in some cases, which may constitute a large resource available to plants if it could be successfully mobilised (Richardson, 2001). A number of possible mechanisms by which microbial P mobilisation can occur has been reviewed by Calvo *et al*. (2014), Owen *et al*. (2015) and Lugtenberg and Kamilova (2011). These include the production of organic acids (Jones *et al*., 1998) which can chelate bound cations,

producing dephosphylating enzymes, and via the dissolution of P-containing minerals (Jones and Oburger, 2010).

In addition to yield increases in crops treated with biostimulants, formulations of plant growth-promoting fungi may also offer additional benefits to crops, which include improved resistance to abiotic stresses (Frioni *et al*., 2018) and pathogen suppression (Calvo *et al*., 2014). The fungi most typically associated with these benefits are arbuscular mycorrhizal fungi and root-associated fungi (Storer *et al*., 2016), but there exist possibilities that plant growth-promoting benefits may be derived from a far greater number of taxa than previously thought (Detheridge *et al*., 2016).

While many studies have involved appraising plant growth-promoting effects of commercial biostimulants, very few studies are considered to have demonstrated direct beneficial effects derived from biostimulants on crops, or to demonstrate beneficial effects besides improved yields, or to present sufficiently robust methodology in their experimental approaches (Jones and Oburger, 2011).

1.2. Aims and objectives

The thesis is divided into eight chapters, beginning with a literature review. These chapters have all been written to achieve the primary aim of examining the use of fungal bioinoculants in agricultural contexts and their potential to improve crop phosphorus uptake.
The specific objectives of the research are as follows:

- To report findings of existing research on the markets, modes of action and areas of necessary research concerning biostimulants (Chapter 2)
- To examine the effects of a number of commercial biostimulants on the growth, health and plant P uptake of an experimental *Lolium perenne* crop in a controlled environment (Chapter 3)
- To explore effects of commercial biostimulants on the growth and plant P uptake of an experimental *Arabidopsis thaliana* crop and the effects of biostimulant amendments on the substrate microbial community in a controlled environment using a phenomics approach (Chapter 4)
- To examine the plant-soil-microbe effects of nutrient amendments and grazing regimes in a field environment using a DNA metabarcoding approach, and to mechanisms and implications which may explain any observed differences in community structure (Chapter 5)
- To synthesise the themes and findings across the studies and identify opportunities for further work within the research area (Chapter 6)

1.3. Thesis synopsis

The experimental work of the study have been presented as six separate papers, and as such there is some repetition of introductory material, methods and references, which is unavoidable in the preparation of a thesis of this type.

A literature review is presented in Chapter 2 which discusses the concepts, mechanisms and processes involved in plant growth-promoting microorganisms and their potential for use as commercial biostimulants. The principles of soil ecology are discussed, and the modes of action involved in plant growth-promotion associated with microorganisms are reviewed. The chapter also explores some of the many shortcomings and limitations for biostimulants, focussing on specific problems with key stages of the development, refinement and establishment of biostimulant formulations, and framing this in the wider context of whether biostimulants work in practice, persistence, spread and negative consequences to the host crop and to the environment. Areas identified as priorities for research are described.

Chapter 3 is an experimental chapter which utilised a high-throughput plant phenotyping platform to examine the effects of some commercially available biostimulants on an experimental *Lolium perenne* crop in terms of growth, health and P uptake. Comparisons are made between morphometric variables to ascertain which treatments were associated with improved growth yields, which include measurements of biomass and leaf and root P levels. Chlorophyll fluorescence spectroscopy was used to examine photosynthetic efficiency in the plant leaves, as well as a calculation of crop water use efficiency over the experimental period. Both of these variables have suggested some insights into the health of plants under different treatments, and the limitations and caveats have been explored critically.

Chapter 4 consists of a phenomics approach to quantify biostimulant effects on an experimental *Arabidopsis thaliana* crop in terms of growth and crop P uptake, in combination with a DNA metabarcoding approach to examine shifts in fungal community structure associated with the nutrient amendments and biostimulants applied. The method develops the approach used in Chapter 3 by using different camera positions and recording a greater number of variables to help interpret biomass accruement and overall influence of the biostimulant on crop morphology. Sterilised controls of all biostimulant treatments are utilised, along with P-amended and non-P amended soils for each treatment. Explanations for differences between treatments are proposed, with the findings being used to suggest modes of action and key indicator species involved in interactions between plants, soils and microorganisms.

Chapter 5 is the final data chapter, which uses a similar DNA metabarcoding approach to the method described in Chapter 4 to examine changes in fungal community structure in field environments under sustainable intensification treatments (consisting of different grazing and nutrient regimes) and contrasted these with business as usual treatments. Explanations for differences between treatments are proposed, and findings are used to suggest a number of possible implications of different management approaches. Possible modes of action of key indicator taxa are suggested. The results are interpreted in the context of what the data may mean for biostimulants, which if to be effective in working towards more sustainable food production must be shown to work in combination with sustainable intensification approaches.

A general discussion is presented in Chapter 6, which offers a synthesis of the key themes and findings of the thesis. The strengths and limitations of the approaches described in this study are discussed critically, and recommendations for further work in the research area are proposed, as are the implications of these findings.

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Chapter 2

The role of plant growth-promoting microbes in agriculture – a review

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2.1. Introduction

In recent decades, the global use of chemical fertilisers in agriculture has increased considerably, which has contributed to pollution of soil, air and water (Vassilev, 2015). This corresponds with an increasing demand for resources from a growing world population, putting increasing stress on the world's food production systems. The need to develop sustainable nutrient management regimes to safeguard food supplies is becoming a matter of increasing importance. One particular concern is management of the supply of phosphorus (P), which is an essential component of diverse organic molecules required for plant metabolism (nucleic acids, ATP and phospholipids; Campos *et al*., 2018). The mineral form of phosphorus used in agricultural fertilisers is a finite and non-renewable natural resource, and after nitrogen, phosphorus is the most limiting nutrient for plant growth. Mineral and organic forms of the fertiliser are susceptible to being dissolved into solution with rainfall, which contributes significantly to eutrophication (Richardson, 2001).

The potential of fungi to be used to improve P mobilisation in agricultural contexts has been well documented (Rouphael *et al*., 2015), and the use of plant growth-promoting microorganisms in the form of bioinoculants in agriculture has been advocated as a valuable component of integrated nutrient management regimes (Ruzzi *et al*., 2015; Vassilev, 2015; Rouphael *et al*., 2015). The commercial exploitation of plant growth-promoting microorganisms in the form of crop bioinoculants has been reported as a potential mechanism for addressing these problems (Ruzzi *et al*., 2015; Vassilev, 2015). Carefully managed formulations of plant growth-promoting fungi and bacteria may be able to facilitate reductions in the volume of inorganic fertilisers necessary for crop production by increasing the bioavaiability of soil P reserves (Ruzzi *et al*., 2016). Phosphorus is highly insoluble even when the element is present in large concentrations, it may not be easily accessible to plants (Fageria *et al*., 2011). The primary factors which limit P availability to plant roots are levels of phosphate ions in the soil and the buffering capacity of the soil in relation to P (Sattari *et al*., 2012). Historic application of P, mainly over the past 50 years has resulted in relatively high P levels in soil (Richardson, 2001), and as such, there exists considerable value in the potential to remobilise this P for the purposes of improved plant mineral nutrition. Various modes of action have been suggested by which microbes used in commercial bioinoculants may be able to mobilise these forms of phosphorus innacessible to plants (Lugtenberg and Kamilova, 2011). The evidence presented for this and the wider context of the use of microbial inoculants is reviewed herein. Both fungi and bacteria are discussed, due to the influence of each on the other in soil microbial communities and soil function.

2.1.1. Environmental consequences of P pollution

The role of phosphorus pollution in freshwater and marine systems has been well established and the effects of eutrophication have been described in detail (Vollenweider, 1970; Ascott *et al*., 2016). Europhication as a consequence of P enrichment of water refers to the resulting bloom of algae or other aquatic plants which disrupt the ecosystem balance and functionality, and ultimately leading to anoxic conditions of the water, and in the UK the trophic state of the majority of lakes and rivers is largely determined by P (White and Hammond, 2009). Since most UK rivers and lakes have elevated P resulting from anthropogenic enrichment, the risk of eutrophication is high (Haygarth *et al*., 2004). In the UK, agriculture is responsible for a considerable proportion of this P flux, calculated by White and Hammond (2009) as being in the range of 10-28% of total P load to UK rivers depending on location. Phosphorus levels in UK rivers has been decreasing considerably over the last fifty years, with recent estimates reporting values at 50% of those in 1974 (Worral *et al*., 2015). This is due to a concerted effort from legislation designed to reduce P inputs to rivers, which in the European Union has been a result of the Water Framework Directive (European Union, 2000), although more improvements are necessary in agricultural practices in order to comply with the directive's new standards (Ascott *et al*., 2016). As such, it is recognised that measures must be taken to reduce P enrichment of watercourses from agriculture (White and Hammond, 2009; Worral *et al*., 2015; Ascott *et al*., 2016), and as such the potential exists for phosphorus mobilising microorganisms in agriculture to contribute to an integrated management solution to reduce the levels of necessary P amendments to soils.

2.1.2. Nutrient stripping

The global overexploitation of soils due to intensive agricultural practices has resulted in a number of consequences, which include erosion, acidification, salination, but increasingly of concern is the permanent loss of macro and micronutrients from the soil (Jones *et al*., 2012). Crop growth and development requires seventeen essential nutrients, which are carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, zinc, copper, manganese, iron, boron, molybdenum, nickel and chlorine (Fageria and Moreira, 2011). Of these, plants obtain carbon, hydrogen and oxygen via the air and soil solution, and constitute around 95% of plant mass. As well as these three elements plants require large quantities of P, potassium, calcium, magnesium and sulfur, which are collectively termed macronutrients.

Nutrient stripping refers to this sustained loss of minerals from soils which lack the capacity to replace them, and the issue constitutes a major threat to the security of world food production. The problem arises from the global inbalance between soil inputs and outputs, which depends

very much on crop type, soil type, climate and wider economic variables (Jones *et al*., 2013). In relation to phosphorus, geologically older and more weathered soils are at a greater risk of stripping, due to the progressive leaching of base cations from the soil over time, although geologically younger soils such as those in Europe are also susceptible (Hedin *et al*., 2013; Bol *et al*., 2016; Veldkamp *et al*, 2020). The stripping of a given macronutrient will drastically reduce the overall use efficiency of other macronutrients (Kering *et al*., 2013; Brar *et al*., 2011; Ameen *et al*., 2018), but in addition micronutrient stripping can have the same effect and prove harder to rectify, due to the difficulties inherent in identifying deficiencies, (Jones *et al*., 2013), as well as barriers to the necessary resources preventing many parts of the world from implementing remedies to the issues.

2.1.3. Finite resources of micronutrients

Micronutrients are elements which are essential to crop growth and development which are required in smaller quantities than macronutrients, and consist of zinc (Zn), iron (Fe), copper (Cu), boron (B), molybdenum (Mo), manganese (Mn), chlorine (Cl), and nickel (Ni), which are all needed for plant growth, and if availability of any of them is limited then plant growth will be reduced (Fageria and Moreira, 2011). Micronutrient deficiencies in food crops have serious wider implications in diets, and is a very common issue in various parts of the world. An estimated 1 billion people globally suffer from malnutrition of trace elements, and strategies for safeguarding food security and improving global health must include improving the bioavailaibilty of micronutrients in food crops (Fageria *et al*., 2012).

2.1.4. Definition of terms

The term “bioinoculants” in this review will be used to describe substances which use microorganisms to enhance plant growth (summarised from Owen *et al*., 2015). This term encompasses a variety of other terms, including biostimulants, biofertilisers and biopesticides. Of these, it is perhaps only the term “biostimulants” which is most rigorously defined. The European Biostimulants Council considers biostimulants to be materials composed of rhizosphere-influencing microorganisms which are beneficial to plant growth by stimulating natural processes, resulting in a variety of positive outcomes, specifically with respect to nutrient uptake, tolerance to abiotic stresses and optimising crop quality (EBIC, 2019).

The term “bioinoculant” shall be used in this review to encompass the above, but will also include the potential for microorganisms to improve plant disease resistance, obtain nutrients from a wider number of sources and improve rooting structure (biopesticides and biofertilisers, respectively). The term “biofertilisers” is best defined by Vessey (2003) as a substance containing live microorganisms, which, when introduced to the proximity of plant roots, will

either colonise them or the rhizosphere, and improve growth through enhanced mineral nutrition. This must serve to distinguish biofertilisers (and in the broader sense, bioinoculants) from organic fertilisers, which in contrast enhance soil fertility by their own decay or by direct provision of nutrients. The term entered scientific literature in the 1970s and has been used increasingly as a description for microbial inoculation (Vessey, 2001).

The manner in which nutrient availability is enhanced is not considered a distinction in this definition. Some organisms can provide both biofertilising and biocontrol effects, for example *Burkholderia cepacia*, which can act as an antagonist towards *Fusarium* spp., a major plant pathogen in arable systems, but in low iron environments is also able to promote plant growth by producing siderophores, which are iron-chelating compounds secreted by microorganisms which can facilitate cell iron uptake (Bevivino *et al*., 1998). The microorganisms that have been exploited commercially for the purpose of improving crop quality in this way include, but are not limited to, arbuscular mycorrhizal fungi (AMF), ectomychorrizal fungi (EMF), (other) root-associated fungi (RAF), intracellular and extracellular bacteria (Kaminsky *et al*., 2019; Fester and Sawers, 2011; Parnell *et al*., 2015). Microorganisms have the ability to mobilise P stores from both inorganic and organic pools (Richardson, 2001), increasing root surface area, and microbial biomass itself is a pool of potentially available P for plant acquisition. The potential for using microbial inoculants to mobilise ‘locked’ P sources is worth exploring.

2.2. Effects and modes of action

2.2.1. Plant mineral nutrition

Root exudates of low molecular weight are readily acquired by microorganisms. The limiting factor for soil microbe population growth is often carbon, supplied in the form of plant root exudates (Lugtenberg and Kamilova, 2009). For this reason, the microbial density in the rhizosphere is several orders of magnitude greater than in bulk soil (Marschner, 2011). Rhizosphere microbes are stimulated root exudates, which in turn lead to increased soil organic matter decomposition (or “rhizosphere priming”; Adamczyk *et al*. (2019)). Relative ratios of rhizosphere microbial density to bulk soil may vary greatly (from 5 to 50), and this may be a result of microbe species, plant age, plant species and plant nutrition status (Marschner, 2011). In spite of the increased proportion of carbon in the rhizosphere, it nevertheless remains relatively limited in nutrient availability, resulting in a highly competitive environment (Storer *et al*., 2016). Many advances have been made into the physical and chemical aspects of soil, but there remain many uncertainties about the dynamics of the relationship between microbial ecology and soil function.

The microbial colonisation of the plant root varies with root surface type. Root meristematic tissue is colonised by soil microbes as it grows. The root exudates enter the soil behind the root tip in the areas of distal elongation, which attracts the most microorganisms. Behind this area, the root hair zones have lower amounts of exudates, and in more mature root zones, the exudates are lower still, and the surface consists principally of cellulose and associated materials less conducive to microbial growth and colonisation. Microbial density and species composition are also affected by the form of carbon exuded. In faster-growing roots, there is therefore a steep curve in the microorganism communities found in the rhizosphere (Marschner, 2011). Endophytes may include bacteria (including actinobacteria) and fungi, all of which can impact growth and nutrient uptake of the plant (Marschner, 2011). As well as decomposing soil organic matter, microbes can solubilise, chelate and oxidise/reduce inorganic nutrients (Figure 2.1.). Microbial biomass in the rhizosphere may also attract predators, such as protozoa and nematodes. Predatory activity releases nutrients from the microbial biomass, thus increasing microbial biomass growth rate (Marschner, 2011).

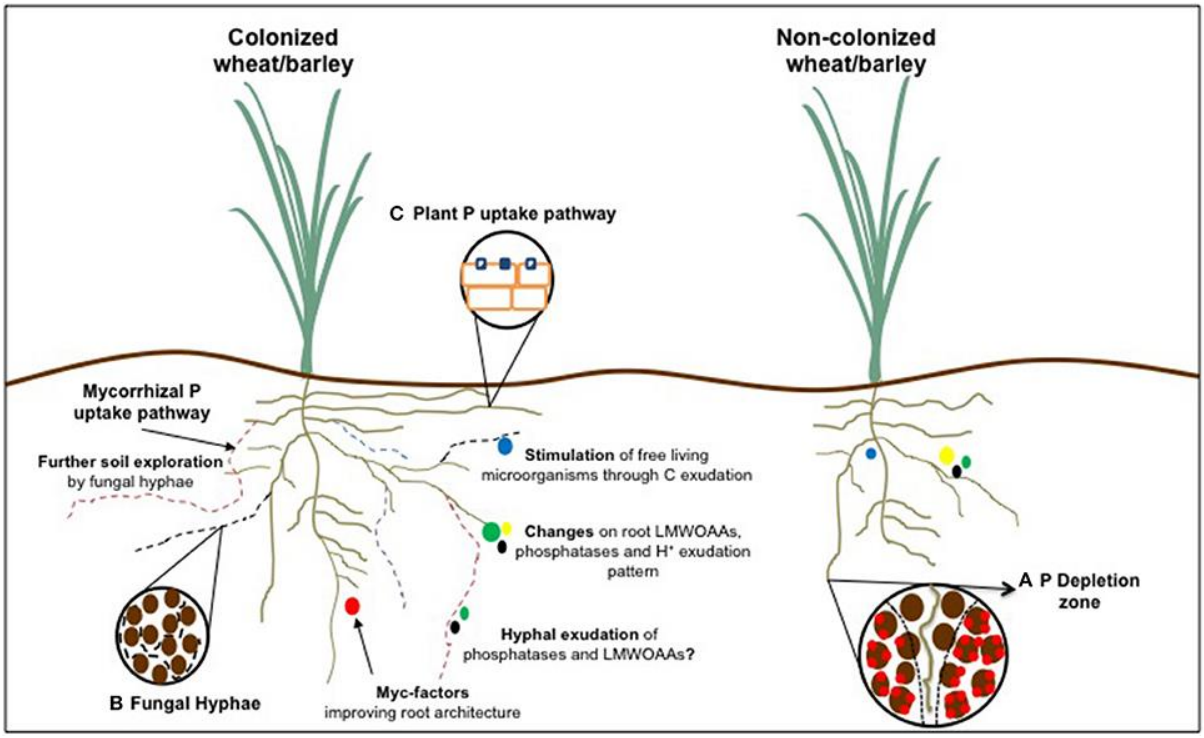

Figure 2.1. Phosphorus acquisition efficiency in plants as enhanced by arbuscular mycorrhizal symbiosis (left) in contrast to a non-colonised control (reproduced from Campos *et al*. (2018)) LMWOAA: low molecular weight organic acid anions.

Changes in microbial density also influence nutrient cycling within microbial biomass – increasing microbial density may increase nutrient immobilisation, whereas decreasing microbial density may result in the net release of nutrients (Marschner, 2011). The abundance of particular strains or species within the microbial biomass is important, as a particular genotype will display particular physiological characteristics (Yu *et al*., 2015). The plant species itself will affect the rhizosphere composition.

The application of fertiliser may also affect the microbial biomass (Yu *et al*., 2015). For example in wheat, the provision of N as either ammonium or nitrate affects the predominance of the pathogenic *Gaeumannomyces graminis* and shifts to a higher proportion of its antagonist, *Pseudomonas* sp. (Bull *et al*., 1991). Phosphorus acts in the same way; by decomposing root exudates, the efficiency of exudates in nutrient mobilisation may be reduced (Marschner, 2011). Microbial density and species composition are significantly affected by fertilisation treatments (Yu *et al*., 2015), and also microbial activity, such as carbon substrate utilisation.

2.2.2. Fungi

Studies of crop responses to fungal inoculation are inconsistent in their findings (Hart *et al*., 2018). While perhaps most studies report improved crop performance (Pellegrino *et al*., 2012; Douds *et al*., 2016), some report no effect (Pellegrino and Bedini, 2014), while others document negative responses following innoculation (Aprahamian *et al*., 2016; Herzberger *et al*., 2015; Janoušková *et al*., 2013).

2.2.2.1. Root associated fungi (RAF)

Root-associated fungi (sometimes referred to as 'non-pathogenic fungi') are well documented to afford benefits for plant growth (Zuccaro *et al*., 2014; Gutjahr *et al*., 2013). The differentiation of RAF from mycorrhizal fungi is not clear-cut but the latter are associated with the formation of distinctive morphological features at the mycorrhizal interface (e.g. arbuscules or Hartig net) and in some cases (e.g. AMF) are obligate biotrophs unable to survive without the plant host. Mycorrhizal fungi are generally much better studied that RAF, and it may be the case that additional study of RAF will reveal the nature of their interactions with plants more clearly (Fester and Sawers, 2011). Carbon exudates from plant roots are conducive to RAF development in the rhizosphere, and as such are easier to use in the form of bioinoculants (Storer *et al*., 2016). Some RAF species promote plant growth by positively altering root morphology as a result of P mobilisation, for example *Penicillium bilii*, which has been shown to increase root length and biomass in low P soils (Sanchez-Evesta *et al*., 2016). This can allow the plant increased resilience to abiotic stresses and enhanced mineral nutrition. Some fungi, such as *Piriformospora indica* (a mycorrhiza-like endophytic Agaricomycetes fungus), are able to promote plant growth and stress tolerance in host plants in a variety of ways (Gill *et al*., 2016). Compounds exuded from the fungi are responsible for stimulating defence and stress responses in plant roots, and can activate plant growth-promoting genes, which enhance plant metabolism (Vahabi *et al*., 2015). Certain species of RAF are particularly well adapted for controlling pathogenic fungi such as *Trichoderma* spp., which is frequently used in bioinoculant products (Mukherjee *et al*., 2013), and is valuable for its ability to colonise plants with no negative impact and being safe to use in proximity to humans and animals. *Trichoderma* also exudes a variety of useful metabolites into the rhizosphere, including peptides and auxins, which stimulate proliferation of root branching and mineral nutrition, positively impacting plant growth (Storer *et al*., 2016). Other RAF species with possible plant growth-promoting effects which have been investigated for the potential to develop into bioinoculants include *Aspergillus, Rhizophagus, Saccharomyces, Mortierella* and *Mucor* (Berdeni *et al*., 2018; Harrison *et al*., 2012; Detheridge *et al*., 2016), and general benefits

attributed to these fungi include improved plant nutrition, increased disease resistance, improved stress tolerance and bioremediation of soil toxins (details summarised in Table 2.1).

Table 2.1: Some important RAF and their growth-promoting effects (summarised from Storer *et al.*, 2016)

Fungal taxa	Plant growth promotion effects	Reference
Trichoderma	Parasitism of fungal pathogens, resource competition with pathogens, induced systemic resistance	Harman, 2006; Harman *et al*., 2004
Penicillium	Mobilise inorganic P by producing organic anions	Richardson *et al*., 2011; Gómez-Muñoz *et al*; 2018
Piriformospora indica	Suspected to interact with phytohormones	Franken, 2012

Storer *et al*. (2016) observe that the crop yield benefits attributable to RAF are variable in nature due to their suspected plant growth promotion mechanisms being mainly increased nutrient uptake and pathogen mitigation, which are only beneficial in nutrient-poor environments or where pathogens are above treatment threshold levels. Storer *et al*. (2016) identify the need for future research where bioinoculant products utilising RAF are examined for plant growth promotion effectiveness in the commercial market.

2.2.2.2. Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizal fungi differentiate from ectomycorrhizal fungi and root associated/non-pathogenic fungi in that AMF are obligate biotrophs (Fester and Sawers, 2011; Smith and Read, 2010; Zuccaro *et al*., 2014), which means that despite their capability to efficiently obtain inorganic soil nutrients and supply them to plants, they require carbohydrates from their host in order to survive and cannot grow apart from them (Gutjahr and Parniske, 2013; Harrison, 2012), and are considered to lack saprotrophic characteristics (Zuccaro *et al*., 2014). Fossil and phylogenetic data suggest that AMF are among the oldest and unchanged organisms in the world, with evidence of mutualisms existing between AMF and the earliest terrestrial plants, which date to the Ordivician period (Fester and Sawers, 2011). AMF have been extensively researched for their potential to enhance plant growth, chiefly for their ability to improve plant phosphorus acquisition (Frew *et al*., 2019), but other effects of AMF in the rhizosphere which are beneficial to plants have now been well documented. These include improved soil stability and structure, improved host plant water status, increased resilience to disease, reduced nutrient leaching and protection from heavy metal poisoning (Asghari & Cavagnaro, 2011; Storer *et al*., 2019). Although there is an abundance of research detailing the plant growth promotion effects of mycorrhizal fungi, the fact that AMF are obligate

symbionts (requiring a plant host for their survival; Smith and Read, 2010) presents difficulties for developing mycorrhizal fungi into commercially viable bioinoculants (Smith and Smith, 2011), leading to most AMF bioinoculants using spores, which will take some time to colonise the plant root system (Storer *et al*., 2016). While this means benefits may not be immediately observable, it suggests that plant growth promotion effects may be sustained over longer periods. The material from which AMF biomass is taken in an inoculant, also known as the propagule, may consist of spore, hyphae or root fragment material. Different inoculant formulations may use different propagules, and the outcome or efficacy of the inoculation may depend on propagule type (Hart *et al*., 2018).

An arbuscular mycorrhizal mutualism involves the development of an extraradical mycelium which extends into the bulk soil, increasing the extent of soil from which the plant may obtain nutrients (Smith and Smith, 2011). In many natural ecosystems, arbuscular mycorrhizal symbioses are of great importance, and can develop mutualisms with over two thirds of all terrestrial land plants (Storer *et al*., 2016). However, the host plant must supply the fungi with carbon, which means that in nutrient-rich soils, the cost of the mychorrizal interaction may outweigh the benefit, or suppress growth. In one such case, Frew *et al*. (2018) reported that AMF inoculation reduced abundances of key plant defence metabolites, reducing plant biomass by nearly 24 % despite plant mineral nutrition being increased (in particular, root concentrations of phosphorus were 50 % higher in AMF-inoculated plants). Nevertheless, the potential for exploiting AMF commercially is of absorbing interest to researchers and a great deal of relevant information has been published on AMF modes of action.

Mychorrizal hyphae are responsible for the transportation of P and other nutrients to plant roots (Marshner, 2012). Mycorrhizal fungi may increase access to other macronutrients, and also afford tolerance to heavy metals and Al toxicity. AMF may contribute to plant mineral nutrition in a number of ways, such as by hydrolysing an insoluble form of phosphorus, phytate, which is the salt of phytic acid. Phytic acid acts as a potent chelating agent, readily binding covalent ions of metals, lowering solubility and therefore preventing adsorption (Howson and Davis, 1983). Some fungal strains can produce extracellular enzymes, which can hydrolyse phytate. This is largely dependent on soil conditions and fertiliser regimes, as excess inorganic phosphorus can inhibit enzyme activity (Sanchez-Evesta *et al*., 2016). AMF species can improve resistance to abiotic stresses. The symbioses formed by such fungal species (e.g. *Gigaspora rosea*) are dependent on lipids secreted by the host (Genre *et al*., 2016). AMF have a large number of genes which are linked to the secretion of acid phosphatases, which may contribute to improved plant P acquisition, but the presence of such exudates in the rhizosphere has only been observed in a small number of circumstances (Campos *et al*., 2018).

Smith and Smith (2011) state that no AMF exists which can be said to enhance the growth of all plants, which suggests that in the context of bioinoculants, “one size fits all” products based on AMF formulations are unlikely to be achievable. The viability of exploiting AMF as commercial crop bioinoculants also faces the challenge of efficacious delivery mechanisms due to AMF being obligate symbionts, and the fact that evidence suggests that AMF induced-responses in plants are observed mainly in P-limited environments (Storer *et al*., 2016). Another caveat inherent in evaluating the evidence around inoculation relates to the lack of studies which will track the progress of the inoculant, documenting whether it has actually established. Without this information, it cannot be claimed robustly that the host crop has benefited in any way from the treatment, with evidence only pointing towards correlation, even if data suggests cause and effect and mechanisms can be proposed (Hart *et al*., 2018). Possible alternative factors could include colonies of alien microbes present in the carrier substrate, nutrient effects from the carrier or perhaps even physical side effects of the method used to apply the inoculant. The method of examining root colonisation of AMF as a proxy for inoculant establishment is criticised by Hart *et al*. (2018), who postulate that such measurements only have meaning in systems which do not have resident fungal communities, as it cannot be discerned which AMF structures result from inoculants and the natural ingress of fungi from the environment. While this is true, measuring root colonisation could still provide indirect evidence for biostimulant establishment.

2.2.3. Bacteria

2.2.3.1. Bacteria in plant growth promotion

Plant growth-promoting rhizobacteria are of major importance in developing bioinoculants, and shall be referred to herein in the broader sense as plant growth-promoting bacteria (PGPB) or plant growth-promoting rhizobacteria (PGPR). The distinction is worth making, since the former include bacteria which colonise the endosphere rather than the rhizosphere, and have vastly divergent properties (Storer *et al*., 2016). The modes of action of PGPB/PGPR are of considerable research interest, and include the ability to increase the rate of root growth and improve root morphology, enhance mineral nutrition, and promote positive interactions with other microorganisms (Lugtenberg and Kamilova, 2011). "Plant growth-promoting rhizobacteria" was first used as a description by Kloepper and Schroth (1978), and has since been of increasing interest to researchers. PGPR may colonise the rhizosphere, the root surface or through dead cell layers (Guo *et al*., 2015). The ability of microorganisms to colonise the rhizosphere is dependent on how well adapted they are to the conditions of the rhizosphere, which may have different pH, O_2 partial pressure, osmotic potential and plant exudates than in bulk soil (Marschner, 2011). Rhizospheric interactions often involve the PGPR fixing itself to the plant surface, though not uniformly (Andrews and Harris, 2000). In some instances, the PGPR will occupy the spaces within plant apoplasts, and is commonly observed among legumes. Intracellular growth has been documented, but is rare (Qianli *et al*., 2001). The process of these interactions is regulated, and involves chemo-attraction, the attachment of the *Rhizobium*, microsymbiont infection and root nodule development where bacteria have colonised (Gualtieri and Bisseling, 2000). In endophytic relationships, the mechanism by which the rhizobium infects the host and the location where it may be found is not clearly understood.

2.2.3.2. PGPR modes of action

A difficulty observed by Vessey (2003) is the high proportion of research focused on PGPR which merely documents yield or biomass increases when a particular PGPR is introduced to a plant culture, but fails to include morphometric data such as root weight, length and morphology, which would help to identify the mode (or modes) of action. At present, the modes of action of PGPR include (but are not limited to) improving plant nutrition, increasing the surface area of roots, entering beneficial mutualistic relationships, competing with pathogens for nutrients and niches, antagonising pathogens and induced systemic resistance (Lugtenberg and Kamilova, 2011).

2.2.3.3. Increasing rhizosphere nutrient availability

Enhanced plant mineral nutrition can be achieved through mineral solubilisation, which improves its availability. This is of particular importance with phosphorus, since it has a high affinity for binding to soil particles and complexes, rendering it inaccessible to plants. PGPR are able to solubilise inaccessible phosphorus in two ways:

1. Some PGRB can produce organic acids, which are able to mobilise insoluble forms of phosphorus by chelating bound cations (Calvo *et al*., 2014).
2. Other PGPR produce phosphatases, which can dephosphorylate organic forms of phosphorus, such as phytate (Rodriguez & Fraga, 1999).

As well as solubilising forms of nutrients which are unavailable to plants, PGPR can produce siderophores, which can help transport minerals, notably iron (Calvo *et al*., 2014).

Large volumes of soil P are unavailable to plants, to the extent that the accessible portion is generally a small fraction. This is due to it being insoluble (Konesky *et al*., 1989), and only soluble forms of P can be absorbed by plants ($H_2PO_4^-$ and HPO_4^{2-}). The principle mode of action for accessing this P source through PGPR is the secretion of phosphatases and organic acids, which can convert the insoluble P forms to plant-available forms (Richardson, 2001; Kim *et al*., 1998). Several genera of PGPR have been shown to be very efficient at solubilising phosphorus, including *Bacillus, Azotobacter, Pseudomonas* and *Mesorhizobium* (Ahmed *et al*., 2008). In the rhizosphere, bacteria which can mobilise phosphorus are not rare (Nautiyal *et al*., 2000; Vázquez *et al*., 2000), but this may not necessarily mean that the organism promotes plant growth. This is suggested by studies which compare growth rates of plants inoculated with phosphorus-solubilising bacteria and do not always find a correlation (Cattelan *et al*., 1999)

2.2.4. Additional modes of action

While PGPR are chiefly involved in enzymic degradation of insoluble nutrients or chelation of bound cations, PGPR may also induce increases in root length, and stimulating the increase in root hair abundance (Vessey *et al*., 2003).

2.2.4.1. Phytohormones

Plant metabolism, growth and defence are controlled by phytohormones, which include indoleacetic acid (IAA), cytokinins, gibberellins, ethylene and abscisic acid (ABA). Plant growth and metabolism is optimal when the relative levels of these phytohormones are balanced. These phytohormones are produced by plants and synthesised by rhizosphere bacteria simulataneously, in identical molecular forms (Forni *et al*., 2017; Lugtenberg and Kamilova, 2009; Storer *et al*., 2016; Ahmad *et al*., 2008). As such, symbioses with soil

microorganisms can influence crop morphology through mediating phytohormone concentration, but whether outcomes of such mutualisms are positive, negative or inconsequential to crop health and yield depend on the specific plant/microbe combination and the environmental conditions (the “mutualism-parasitism-continuum”; (Mandyam and Jumpponen, 2015). The most well-researched phytohormones is IAA, which is involved in the regulation of cell division, differentiation and elongation. IAA helps plants alleviate abiotic stresses, has a role in triggering the germination process, and stimulates root proliferation (Ahmed *et al*., 2008). In combination with other phytohormones, the levels of IAA controls plant responses to light and gravity, as well as pigmentation. Another key group of plant hormones are cytokinins, which also regulate cell division, differentiation and elongation. In addition to this, cytokinins prevent early sensescence, and reduced levels of cytokinins induce stomatal closure, lowering carbon import and consequently crop yield (Kaushal *et al*., 2016). Whilst bacterially synthesized cytokinins have been shown to improve growth of plants under drought stress, their functionality and modes of action in relation to crops remains poorly understood (Forni *et al*., 2017), and in any case, since plant metabolism is optimal when there is a balance between the levels of various phytohormones, microbial inoculants introduced for the purpose of increasing these levels may not actually afford any improvement to their relative concentrations and as such may not deliver any useful outcomes.

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Careful ammendments of IAA and other phytohormones can in theory be useful for stabilising this balance. One such example would be that they can increase the synthesis of gibberellin, a key phytohormone involved in the regulation of growth and germination that can be synthesised by microbes, and which has direct consequences on yield and mineral use effiency, as well as nitrogen metabolism. In terms of improving crop resilience to abiotic stress, gibberellin is involved in the mediation of salt stress and drought stress. Gibberellin also facilitates "crosstalk" between other phytophormones, which again relates to the importance of maintaining the balance and ratios between phytohormones, the relative levels of which being optimal mediators of plant growth. This demonstrates why merely supplementing the rhizosphere with an excess of phytohormones will not necessarily deliver any positive outcomes, as it is the relative concentrations between the phytohormones as opposed to the magnitude that ultimately have an effect on yield.

Microbially synthesised phytohormones can also be involved in the mitigation of phytohormones which downregulate growth. An example of such would by several PGPR which are able to produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can degrade the precursor to the phytohormone ethylene, the presence of which may inhibit the growth of root tissue (Glick *et al*., 1998, Holguin and Glick, 2001). This mode of action appears to be most effective when plants are subject to drought or salt stress, as this tends to be when ethylene is most frequently produced. Again, this research is often subject to the caveat that evidence is still very limited as to whether beneficial effects from PGPR-synthesised phytohormones observed in laboratory conditions translate into yield benefits in field environments, where additional abiotic stresses, competing microbial communities and unknown concentrations of rhizosphere phytohormones make outcomes much more unpredictable.

2.2.4.2. Disease resistance

In addition to abiotic stresses, crops face biotic stress from pests and pathogens, and plants have various mechanisms of responding. This can take the form of local responses in the affected part of the plant, but also include effects across the entire organism (Romera *et al*., 2019). These effects are Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR). ISR is a response stimulated by root-associated microbes and fungi, which can improve plant immunity to disease or insect pests. SAR is a response directly elicited by pathogen pressure on the plant. ISR can be elicited in plants due to determinants being present in the bacterial culture being introduced. These may act as triggers or signals which activate the plant's latent resistant responses. Chief among these are lipopolysaccharides present in the outer membrane of certain PGPR, which have been demonstrated to act as

triggers for inducing latent resistance (Leeman *et al*., 1995). Of the PGRP which have been well-researched, *Pseudomonas* spp. appears to be particularly effective at eliciting ISR in plants (Rana et al., 2020; Mhatre et al., 2019; Wei *et al*., 1996, Ramamoorthy *et al*., 2001). This can be inferred as a result of inoculated plants demonstrating resistance when pathogens are introduced, rather than merely show increased yield. A tradeoff exists, therefore, between the perceived benefit to a crop from inducing resistance by microbial means and the significant metabolic cost to the plant involved in this response, which may translate to a yield loss. The pathogen pressure of the environment will vary, and even in areas where it is high, microbial inoculants applied with the intention of eliciting ISR could also translate into yield loss, as microbes may compete with plants for certain nutrients (Romera *et al*., 2019).

Some PGPR can produce substances which have toxic effects on plant pathogens. These can include hydrogen cyanide (Storer *et al*., 2016), pyrrolnitrin and pyocyanine (Ramamoorthy *et al*., 2001). These antibiotics are secreted to the rhizosphere, and their concentration can accumulate, and reach a level which prevents pathogen growth (Berendsen *et al*., 2012). Certain PGPR are able to confound potential pathogenic activity by degrading signalling molecules used by plant pathogenic bacteria (such as homoserine lactones, or AHL), which are suspected to contribute to the pathogen biofilm. In small concentrations, some PGPR can inoculate and the plant against disease by stimulating the plant's latent resistance, in what is sometimes referred to as acquired physiological immunity or translocated resistance (Bhattacharyya and Jha, 2012; Ramamoorthy *et al*., 2001), although this also is associated with a metabolic cost to the plant (Romera *et al*., 2019, rendering the overall effect negative if the pathogen pressure of the environment is not particularly severe. The stimulatory effect of the PGPR acts as an inducing agent activating intrinsic processes of pathogen resistance within the plant, which can then be expressed in the incidence of a pathogen infection. This may have a very significant effect, immunising the plant against possibly several plant diseases (Compant et al., 2010; Compant et al., 2005; Wei *et al*., 1996). Case studies have shown this to be observed in field conditions (Mhatre et al., 2019; Bhattacharyya and Jha, 2012**;** Wei *et al*., 1996).

Siderophores and salicylic acid can act in similar ways (Mhatre et al., 2019; Ramamorthy *et al*., 2001). A similar mechanism is known as systemic acquired resistance (SAR), which differs in that it can be induced by both biotic and abiotic activating agents. Biotic inducers may include virulent pathogens as well as non-pathogenic microbes, and fungal cell wall metabolite elicitors (Rasmann et al., 2017;.Ramamoorthy *et al*., 2001). Abiotic activating agents may include salicylic acid and ethylene. Some PGPR can produce sidephores, which can prevent the uptake of Fe ions by plant pathogens, which can hinder their effects. A notable example is pseudobactin, a fluorescent sidephore produced by certain strains of the rhizobium

Pseudomonas. It contains a hydroxamate ligand, D-N5 –hydroxyornithine, which has been shown to be responsible for mitigating the growth of the phytopathogen *Erwinia carotovora* in soils of low Fe status (Ambrosi *et al*., 2000). By colonising plant roots, PGPR may be able to simply block pathogen colonisation by preventing them accessing entry points to the host (Rasmann et al., 2017; Lugtenberg and Kamilova, 2009).

PGPR may be able to colonise the pathogen itself, thus hindering its growth. An instance of such hyperparasitism is *Pasteuria penetrans* involved in the control of root-knot nematodes (Gupta & Vakhlu, 2018). As much as 20 % of photosynthesised carbon from plants can be deposited into the rhizosphere as low molecular weight exudates, which provides conditions favourable to the growth of bacteria and fungi (Lugtenberg and Kamilova, 2009). It is now well supported that a variety of rhizobia have plant growth-promoting properties, and can be directly or indirectly involved in bringing about increases in yield, heightened mineral nutrition, and also increased pathogen and stress tolerance (Lugtenberg and Kamilova, 2009; Storer *et al*. 2016).

2.2.5. Metabolic costs of inoculation

For a plant to form a mutualism with any plant-growth promoting microorganism, a significant metabolic cost is involved to the host, which will to a greater or lesser extent translate into yield loss. This is due to the nature of mutualisms typically requiring carbohydrates from the host in exchange for some benefit from the microorganism, and depending on a number of factors, the cost of the former may exceed the benefit of the latter, resulting in no net nutritional, physiological or metabolic benefit to the host. The effects of the metabolic cost of inoculation in host plants has been observed and documented in recent research. Berdeni *et al*. (2018) explain an absence of beneficial effects observed in the tissue of hosts inoculated with AMF as a consequence of down-regulation of plant nutrient uptake resulting from host colonisation, which leads to no overall benefit or nutritional gain from the inoculation to the host. Microbes also compete with plants for some nutrients in the rhizosphere, and in so doing can negatively impact plant mineral nutrition (Romera *et al*., 2019).

2.3. Context of bioinoculant market

At present, the market for bioinoculants is mainly composed of N-fixing products, but it is predicted that P-mobilising products are likely to see increased demand (Kaminsky *et al*., 2019; Owen *et al*., 2015). Sources of mined rock phosphorus are finite, and estimates concerning projections for supply exhaustion range from 100 to 400 years, with supplies primarily mined from outside Europe (Scholz *et al*; 2013 Jones *et al*., 2012). Most soils in the UK are to some extent phosphorus deficient, and therefore mineral forms of P are often used to enhance crop production. It is these mineral forms of the fertiliser which have the highest eutrophication potential (Richardson, 2001). The global market value of bioinoculants was most recently estimated at $1 billion (ADAS, 2016) and the market growth rate is projected as 13.4 % for the 2019 – 25 period (Adroit, 2019). The largest section of the global market is Europe, predicted to reach a value of $1 bn as of 2020; with 3 million ha of European grasslands having had biostimulant treatments applied (EBIC, 2019). EBIC estimates the EU sector of the biostimulant market to be increasing by 10 – 12 % annually (EBIC, 2019).

2.3.1. Regulation

As of June 2019, the EU has recognised biostimulants as a separate category of fertilisers. The Single Market will see its first EC-marked biostimulants in July 2022 (EBIC, 2019), although the specific criteria for implementation measures are not currently addressed. Section 22 of Regulation (EU) 2019/1009 in the Official Journal of the European Union classifies plant biostimulants as eligible for CE marking (amended Regulation (EC) No 1107/2009). Section PFC 6 details thresholds for micronutrients and contaminants, and stipulates that the biostimulant product shall “have the effects that are claimed on the label” for specified crops. Section PFC 6(A) restricts the presence of various hazardous microorganisms in microbial biostimulants, including *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. In terms of quality control, the natural diversity of bacterial and fungal species may result in variability in their functions, which may lead to unpredictability of performance, which is exarcerbated if the products are not stored in conditions conducive to maintaining microbial viability (Owen *et al*., 2015).

2.3.2. Preparation of bioinoculants and carrier media

The methods used in preparing commercial bioinoculants vary considerably, and depend on the organism. For instance, root-associated fungi-based formulations do not require a living plant host during the preparation stages, whereas arbuscular mycorrhizal species do (Storer *et al*., 2016). As such, mycorrhizal bioinoculants are more likely to use spores than cultures of the fungal material (Frew *et al*., 2019). With regards to bioinoculants which utilise bacteria, batch-fed culture methods are often used on high densities of microbial cultures (Chaudhary

& Shukla, 2019). Using molecular diagnostic and culturing methods, plant growth-promoting microorganisms can be isolated and sub-cultured into single colonies and pure cultures (Parnell *et al*., 2015; Kaminsky *et al*., 2019), which can then be developed into various bioinoculant carrier media, which may include granules, wettable powder, or liquid suspensions. Bioinoculants currently commercially available often contain carriers intended to optimise rhizosphere conditions, such as DAP or denitrifying bacteria-inhibiting minerals (Rouphael *et al*., 2015).

Depending on the method of maintaining colonies used in biostimulant preparation, formulations may undergo genetic bottlenecks, which could result in biostimulants having lower genetic diversity than native soil microbial communities, which could affect both biostimulant viability and persistence (Hart *et al*., 2018). Biostimulant development must also undergo a lengthly process, which include multiple stages of collection, isolation, refinement, culturing, production, storage, application, establishment and legacy. The microbes must survive the whole process, from the preparation stages to the establishment ones, where microbes must then compete with existing biota to establish themselves. Generations of microbes may pass during this process, which as well as creating possible genetic bottlenecks may also lead to adaptation and change in functionality.

2.3.3. Bioinoculant mode of application and longevity in soil

Due to the relatively recent emergence of bioinoculants for commercial use, and the fact that they were originally developed for use in horticultural rather than outdoor arable contexts, the long-term effects in arable and grassland environments has yet to be properly studied, and is an important consideration for future research (Storer *et al*., 2016). One obvious difficulty is that a high diversity of bacteria will be present in field soils (Detheridge *et al*., 2016), and as such, introducing more bacteria may not necessarily afford any tangible effects.

An observation which can be made at the outset is that the ecological function of the microorganisms used in the bioinoculant will dictate to a certain extent the longevity of any treatment effects in the soil. AMF structures may take some time to grow, but once established, their effects may continue throughout the growth season. Despite the longer time period for spore persistence in soil than mycelium fragments, spores are slower to colonise plant host, and as such, commercial bioinoculants will often include both spores and fragments (Frew *et al*., 2019). Du Jardin (2015) observes that unlike chemical treatments, the effects of microbial inoculants may take some time to be induced, and therefore a key area for future research involves the combined effect of bioinoculants with fertiliser inputs. From this, it could be argued that introducing biostimulant as a form of seed pelleting allows early establishment

of desirable taxa, and may therefore afford more direct benefits than adding ammendments at the field scale.

2.3.4. The effectiveness of bioinoculants: discrepancies in scientific literature

When considering how to maximise the efficacy of a bioinoculant approach to crop production, Smith and Smith (2011) observe that it is unlikely that any specific species will afford universal benefits to all plants in all circumstances, soil conditions or soil types. In addition to this, there is reported to be a lack of consistency between batches of bioinoculants, which leads to varied results in bioinoculant effectiveness. Concerns raised about the quality of scientific literature examining bioinoculants often observe that lab-based trials frequently produce more positive results than field-based trials (Storer *et al*, 2016; Owen *et al*., 2015). This may be a result of many biotic and abiotic factors, and which may influence the efficacy of the bioinoculant. For instance, plant growth promotion as a consequence of microbial inoculation may not necessarily be associated with characteristics such as P solubilisation, which are observed under laboratory conditions (Sanchez-Evesta, 2016). A variety of factors may be involved in these discrepancies, and the matter remains a key area for future research (Hart *et al*., 2018). Gómez-Muñoz *et al*. (2018) observe that increased plant growth attributed to inoculation with *Penicillium bilaii* in literature may have been facilitated by increased phosphorus availability, but that similar results are observed when soil P is not a limiting factor, adding evidence to suggest that more mechanisms are involved. The study by Gómez-Muñoz *et al*. (2018) into the effects of *Penicillium bilaii* on maize growth suggests that although root morphology and function can be stimulated by fungal inoculation, but that effects are dependent on rhizosphere nutrient status, since few effects were observed when other nutrients were limited in the trial.

One major caveat for over-interpreting the findings of field studies into biostimulant outcomes is that many such studies tend to cluster around either marginal or extremely limited nutrient status soils (where the likelihood of positive outcomes following inoculation is magnified) or in a series of conventional agricultural soils of low inoculum potential, whereby the benefits of applying biostimulants may not offer scope for any obvious benefits (Hart *et al*., 2018). It is therefore important not to overstate results from study sites of abnormal or extreme soils, which may produce findings of limited applicability to the more common systems.

It also seems likely that growth responses of crops in field environments which are attributed to fungal symbioses are not necessarily linked to improved P acquisition (Campos *et al*., 2018). A meta-analysis of wheat responses to inoculation with AM fungi (Pellegrino *et al*., 2015) notes that yield and P accumulation correlate positively, while biomass only correlated weakly with AMF colonisation rate. Campos *et al*. (2018) also observe that the impact of any identified modes of action associated with inoculating crops with plant growth-promoting fungi

is difficult to quantify, as the competing effects of other plants and microorganisms will influence non-sterile conditions in field environments. Many field studies which explore microbial community change focus on fungi within plant roots and rhizosphere (Santos-Gonzalez, 2007; Dumbrell *et al*., 2011). In more stable bulk soil, reduced root exudate influence may be observed, which in agricultural soils follows a gradient, with less influence on microbial diversity (Wasaki *et al*., 2005; Marilley *et al*., 1998; Kandeler *et al*., 2002), whereby reduced bulk soil nutrient availability permits the proliferation of less dominant species.

A principal cause of discrepant responses of crops to inoculation is batch provenance. The identity and cultivar of the isolate used in the inoculant formulation may result in varied responses in the host, or indeed different outcomes (Buysens *et al*., 2016; Hart *et al*., 2018; Douds *et al*., 2016). Available evidence on to the extent cultivar differences account for these discrepancies suggest that isolates of local provenance result in more positive outcomes (Davidson *et al*., 2016; Pellegrino & Bedini, 2014). For example, Maltz & Treseder (2015) report that inoculant formulations from local provenances outperform commercial biostimulants in remediation of degraded soils. In any case, these outcomes are heavily dependent on inoculant application timing, seasonal effects, tilling, disturbance and fertility factors in the soil, which all increase the difficulty inherent in making reliable predictions and forecasts of outcomes of inoculant benefits for crops (Hart *et al*., 2018).

2.4. Knowledge gaps and priorities for research

Despite the tremendous volume of research being carried out in this area, a number of major questions remain unanswered, with existing evidence remaining vague and inconclusive. In particular, much is unknown about the timing effects on inoculation outcomes, and whether these effects act in combination with abiotic ones. Much remains unknown about the lifespan, dormancy and viability of biostimulants, as well as the most appropriate methods of storage. Optimal application rates of inoculants still remain a matter of discussion, as well as the environmental and site effects (e.g., soil type and management) on the inoculant and its functionality. While it is being increasingly advocated that biostimulants should be tailored to specific crops and specific sites, it is unclear how this can actually be done, and how a cost effective method can be used commercially. The potential of coating seeds or pre-inoculating plants prior to planting is recognised, but little explored. The effects of inoculation on existing microbial community structure still remains unclear despite much investigation, as well as whether there are legacy effects from inoculation. It is also unknown how inoculation affects the structure and composition of plant communities, and to what extent genetic material is exchanged. It is unknown to what extent inoculation provides effects that would not have

arisen from natural ingress of microbes from the environment, and the factors that govern the proliferation and spread of inoculants are also unknown (Hart *et al*., 2018; Ruzzi *et al*., 2015; Vassilev, 2015; Rouphael *et al*., 2015).

2.5. Summary

Whilst the importance of the relationship between agricultural management practices and soil microbial communities is well recognised (Di Salvo *et al*., 2018, Detheridge *et al*., 2016), the processes which determine bioinoculant viability or efficacy remain unknown, and insights into these dynamics could lead to improved robustness of agricultural management decisions (e.g. nutrient management regimes). Research which has explored rhizosphere function suggests that more understanding is needed of the nature of the interactions between plants, soils and the microbial populations (Compant *et al*., 2010). Other research has observed that microbial inoculant efficacy is directly linked to its ability to continue to provide its functions in varying and harsh conditions, specifically pH, temperature and ion concentration differences (Lugtenberg and Kamilova, 2009). The same review observed that the ability of products to meet this challenge is usually unsuccessful, and again made the case for continuing research into microbial inoculant interactions with plants and soils.

The robustness of the evidence-base for bioinoculant efficacy does have issues, and in particular is the high probability of a positive lab-based result for testing the efficacy of a given substance or organism being followed by a less positive field trial result (Storer *et al*, 2016; Owen *et al*., 2015). Du Jardin (2015) asserts that research must seek to characterise the effects of bioinoculants on a wide range of plant crops, and makes a case for using high-throughput plant phenotyping platforms for understanding their modes of action, and describing their interactions with environmental stress factors.

2.6. References

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Chapter 3

Using plant phenotyping methods to assess the efficacy of plant growth-promoting biostimulants on *Lolium perenne*

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3.1. Abstract

Biostimulants containing plant growth-promoting microorganisms have in recent years been proposed as a component of sustainable nutrient management regimes in agriculture on account of their reported potential to reduce the required volume of chemical fertilisers applied to crops. This approach is advocated in the context of increasing demand for resources from a growing world population, creating increasing stress on the world's food production systems. This study sought to quantify the efficacy of seven commercially-available biostimulants purported to improve plant mineral nutrition. Growth trials in controlled environments were designed to examine their effects on *Lolium perenne (L. perenne)* physiology, nutrient acquisition, growth morphology, and plant health. This approach included phenomics methods to examine morphometric parameters of treated plants. There were significant increases observed in leaf area, biomass and morphology of plants treated with an AMF based biostimulant and a rhizobacterial based biostimulant, which also correlated to improved availability of phosphorus in experimental soils and in the leaf and root tissues. The increased P was largely introduced in the form of the biostimulant carrier, suggesting that mineral effects may have greater influence over *L. perenne* growth in the described conditions than microbial effects. Water use efficiency and non-photochemical quenching measurements may also offer evidence that plants treated with these biostimulants could be more resilient to abiotic stress, although this requires further work with added stress variables to confirm whether this is the case. The study was able to demonstrate a method for quantifying biostimulant efficacy, offering indication of the behaviour of key morphometric parameters which model growth changes in crops, substantiating this with measurements of crop P uptake in plant tissues.

3.2. Introduction

Globally, there has seen a substantial increase in the use of chemical fertilisers in agriculture, and this is strongly associated with nutrient pollution of soil, air and water (Vassilev *et al*., 2015). Projected increases in the global population over the coming decades may exacerbate such trends as the demand for food, fibre and fuel grows. Of particular concern in global nutrient management is phosphorus fertiliser, which, after nitrogen, is the most limiting nutrient for plant growth. Mined rock P fertiliser is an unrenewable resource, and is highly susceptible to being dissolved into solution with rainfall, and being a major contributor to eutrophication (Richardson, 2001). The use of plant growth-promoting microorganisms in the form of biostimulants in agriculture has been advocated as a valuable component of integrated nutrient management regimes to reduce such problems (Ruzzi *et al*., 2015; Vassilev *et al*., 2015). Commercial biostimulants are being adopted on a large scale, with a global market value projected to reach $2 billion by 2020 (Adroit, 2019). Biostimulants may offer various opportunities to address nutrient management issues, and may also have cost advantages over other approaches. Much of the current research into biostimulant potential is focussed on arable crops, and the evidence base for using biostimulants for grassland improvement remains to a large extent unexplored. Over 40% of the world's terrestrial area (minus Greenland and Antarctica) is grassland, totalling 52.5 million km^2 (World Resources Institute (2000), based on IGBP data), and 26% of this ice-free terrestrial area is used for livestock grazing (FAO, 2012), constituting a major resource for food production and offering much scope for nutrient management improvement. The use of biostimulants in agricultural contexts have in recent years seen a rapid increase, which is due to a number of purported plant beneficial effects (Calvo *et al*., 2014). Integration of biostimulants to nutrient management systems is proposed to reduce the need for inorganic fertiliser inputs via improved plant mineral use efficiency and acquisition (du Jardin, 2012). However, no consensus has been reached regarding biostimulant efficacy, and even in products that have consistently been demonstrated to be significantly associated with improvements in crop yield, the modes of action responsible for these benefits has never been fully elucidated (Calvo *et al*., 2014; Owen *et al*., 2015). It remains unclear how biostimulant inoculation positively affects the interaction between crops and soil microorganisms and whether this interaction leads to improve yields and stress tolerance (Storer *et al*., 2016). Of the proposed mechanisms of enhancement associated with biostimulants, their potential to mobilise recalcitrant phosphorus reserves within soil, and in so doing contribute to improved plant mineral nutrition, has attracted a great deal of attention, due to dwindling reserves of the mineral form of P (Richardson, 2001). Some well-documented processes by which microorganisms may improve P availability include the production of the enzymes phosphatase and phytase, siderophore production and secreting

organic acids (Storer *et al*., 2016). For the potential of biostimulants to be further developed, there is a need to better understand the modes of action by which they may enhance plant growth (Calvo *et al*., 2014). Fungi-derived plant growth-promoting effects may include positively altering root morphology and increased plant nutrition through mobilisation of existing soil phosphorus reserves (Sanchez-Evesta *et al*., 2016), which could increase root length and biomass in soils of low P availability. It is well documented that microbial biomass in the rhizosphere may release plant-available nutrients in a variety of ways – as well as decomposing soil organic matter, microbes can solubilise, chelate and oxidise/reduce inorganic nutrients, which converts them to a plant-available form (Marschner, 2011). A number of fungal genera commonly used in biostimulant formulations (such as *Penicillium*) are associated with P mobilisation via organic acid secretion, which can chelate bound cations, and various other common fungal genera (such as *Rhizophagus* and *Trichoderma*) are reported to produce phytohormones. Some of these root-associated fungi (notably *Trichoderma*) have been found to directly contribute to improving tolerance to abiotic stress in host plants (Waller *et al*., 2005), and Storer *et al*. (2016) identify arbuscular mycorrhizal fungi and root-associated fungi purported to improve drought stress tolerance in host plants (Romera *et al*., 2019; Santaniello *et al*., 2012). In spite of this, much still remains unknown about the modes of action by which plant growth-promoting rhizobacteria and fungi positively affect plant physiology (Calvo *et al*., 2014; Owen *et al*., 2015). An issue with studies concerned with examining biostimulant efficacy and yield responses under favourable growth conditions is that such approaches are unable to observe potentially improved abiotic stress tolerance effects associated with the biostimulant. These may include drought stress, extreme temperatures or soil conditions such as pH or nutrient status (O'Callaghan, 2016).

Du Jardin (2015) asserts that research must seek to characterise the effects of biostimulants on a wide range of plant crops, and makes a case for using high-throughput plant phenotyping platforms for understanding their modes of action, and describing their interactions with environmental stress factors. A controlled environment setting for an experimental process for measuring the effects of biostimulants that are ultimately destined for use in the field has some obvious disadvantages, principally being that such lab-based trials offer limited insights into the natural environment, where a much greater range of biotic and abiotic effects will govern whether or not biostimulants will be efficacious (Santaniello *et al*., 2012; Kaminsky *et al*., 2019; Hart *et al*., 2018). However, it does offer investigators the ability to isolate mechanisms, which may allow insights into individual effects and modes of action (Neilson *et al*., 2015; Malinowska *et al*., 2017; Duan *et al*., 2018). After a mechanism has been established, further experimental variables can be introduced which will offer more insights into interactive effects, and may be

of importance in helping steer the direction of future research (Honsdorf *et al*. 2014; Duan *et al*., 2018).

In field conditions, there are many variables which govern establishment and efficacy of biostimulants, which include priority effects and niche overlap (Santaniello *et al*., 2012; Hart *et al*., 2018). A biostimulant will have limited efficacy if the microbes introduced are competing with native microbes for the same metabolic niche (O'Callaghan, 2016). Whilst greater understanding of the details of niche overlap and priority effects is of crucial value in evaluating biostimulant efficacy, by removing these variables from the experimental soil substrate, insights into the chemical and biological modes of action of biostimulants may be better observed and quantified (Duan *et al*, 2018; Petrozza *et al*., 2014). Any conclusions that would be drawn from such data must appreciate this caveat, and it must be understood that such conclusions would be purely indicative without verification from further field studies. The phenomics approach has previously been used successfully to demonstrate beneficial effects from commercial biostimulants (Santaniello *et al*., 2012). Petrozza *et al*. (2014) were able to demonstrate using a phenomics approach that a commercial biostimulant (Megafol®) reduced drought-stress related damage in an experimental tomato crop by increasing plant biomass, and by using chlorophyll fluorescence as a mechanism for physiologial stress response. They also reported that the biostimulant treatment was responsible for inducing genes involved in drought stress responses.

Visible light imaging technology, due to low cost and ease of implementation, can be used for plant imaging. Through recording similar wavelength perception as in human vision (400-700 nm), 2D photography can detect useful information for the analysis of leaf biomass, yield and leaf morphology. Another well-developed method which offers insights into plant health, nutrient use efficiency and resilience to abiotic stress is chlorophyll fluorescence spectroscopy, which is based on measurements of re-emitted light from plant leaves under various light-adapted, dark-adapted and saturation intensity light phases. From these measurements, overall plant productivity can be inferred. The technique has been shown to provide insights into plant physiological processes at cellular resolution (Quilliam *et al*., 2006). Leaf chlorophyll is a pigment–protein complex located within the reaction centres in photosystems I and II. When chlorophyll molecules absorb light, the energy can be used in photochemistry, emitted as heat, or a small proportion re-emitted as light. The latter quantity is fluorescence (typically 1–5 %, Quilliam *et al*., 2006), the magnitude of which can be used to quantify the efficiency of photosynthesis and by extension overall plant productivity (if a linear relationship can be assumed between the variables). By measuring chlorophyll fluorescence, inferences can be made as to the proportion of light energy that light-harvesting centres of plants are able to utilise, and as such the productivity of photosystem II is directly related to

assimilation of carbon and overall plant productivity, with the rate of induction of photosystem II electron transport demonstrated to be a coefficient of carbohydrate import by Meng *et al*. (2001). Measuring light-response curves has been demonstrated to be a robust approach to revealing the range of plant physiological plasticity, which are not related to the response of a plant to any given ambient light conditions (Rascher *et al*., 2000).

The aim of our study was to use such technology to quantify morphological differences between plants treated with biostimulants, and in so doing, isolating the modes of action responsible for plant growth-promotion. The methods used herein to characterise the morphological phenotype of a given species (phenomics) have been developed to overcome restrictions of traditional plant morphology measurement methods, affording more accurate quantification of plant growth (Duan *et al*., 2018; Pavicic *et al*., 2017). These techniques utilise image sensors, imaging analysis and robotics. The capability of this infrastructure to facilitate high-throughput and large-scale, non-invasive phenotyping may be used in addition to reduce the time and inaccuracy of traditional measurement methods, which are often manual. The study also adopted a pulse-amplitude modulated photosynthesis yield analysis method involving light-curve programmes on an experimental *Lolium perenne* crop. This consisted of short intervals of increased light intensity and recording instant light-response curves, whilst subjecting experimental crop leaves to dark-adapted phases to ensure that photosynthesis occurs in steady state. The study sought to test the efficacy of a number of commercial biostimulants on a *Lolium perenne* experimental crop and ascertain differences in growth morphology associated with biostimulant treatments.

3.3. Materials and methods

3.3.1 Experimental design

Lolium perenne (*L. perenne*) was selected as the experimental crop for this trial on account of its economic importance in global grasslands (Owen *et al*., 2015). Unlike many crops, growth of *L. perenne* can be measured over the course of several non-destructive cuts (cutting the plant 3 cm above the base of the soil, which allows it to regrow), which permit repeat measurements of plant growth metrics. Seeds of *L. perenne* (cv. “AberMagic”, Germinal, UK) were sown in 360 cm^3 pots (n = 60, eight seeds per pot) in a constant weight of low nutrient compost (peat + lime for pH adjustment/ buffering, WI13, Bulrush, Co Londonderry, NI) and sterilised sharp sand at a ratio of 2 : 1 (Tables 3.2a and b show elemental analysis). To prepare the nutrient conditions, compost used for 55 of the 60 containers was blended with 0.092 g ground Triple Super Phosphate fertiliser (47 % P), in accordance with national recommendations for grass production on low P soils (P index of 0, between 0–9 mg l^{-1});

meaning each container received 0.043 g of plant-available P (equivalent to 100 kg ha^{-1}, Nutrient Management Guide RB209, 2019). The remaining five containers were left as controls without additional P.

Eight unsterilised *L. perenne* seeds were sown on the soil surface of each growth pot (directly sown, not pre-germinated), and the biostimulant treatment was applied in powder form to the entire surface area of the potting substrate one week after sowing, and watered into the soil using approx. 100ml of water per pot. One week following biostimulant application, a non-destructive cut was carried out on all plants (to 3 cm height) to facilitate growth homogeneity. The biostimulants were Biagro MP (MP), Biagro Brassika (KGB), Humostart Rhizobium (UR), Humostart Mycorrhizae (UM), SMFT (MycoForce Fertiliser Treatment), SMMT (MycoForce Mycorrhizal Transplanter) and SMGS (MycoForce Mycorrhizal Grass Seed Coat), applied at approx. ×400 recommended application rate, which was to account of the fact that the small volumes of the recommended application rates which would not have been practical for the surface area of growing pots (Table 3.1c shows application rates). There were two controls: with/without P fertiliser addition, but no biostimulant (Control, Control-P). There were also a sterilised control treatments of three biostimulants, UM, MP and SMMT, which are labelled as UM(S), MP(S) and SMMT(S) respectively. The method of sterilising the biostimulants consisted of autoclaving them (121 °C, 100 kPa, 90 minutes) to help ascertain nutrient effects when contrasted with the live treatments. This gave a total of twelve treatments (in replicates of five). The 60 pots were placed into twelve trays (five pots/tray to allow sideview imaging) on a conveyor system (Photon Systems Instruments, Drazov, Czech Republic). The positions of the pots on the conveyor system trays was completely randomized, with positions assigned using a random number generator. Automated watering was provided on a daily basis to maintain a target of 75 % field capacity (assessed gravimetrically). Compost samples were saturated to determine field capacity and dried to determine dry matter content, and water content was presumed to be the difference in mass between these two values. The target watering mass was calculated based upon a percentage of the water content. Growth conditions consisted of 18 °C day temperature and 15 °C night temperature. Day-lengths were maintained at 14 h using 600 W sodium/LED lamps (intensity = 260 µmol m^{-2} s^{-1} PAR). The pots were placed in custom built trays resting on individual supports, which kept the pots elevated from the bottom of the tray and preventing cross-contamination between pots. During the week after planting, each pot received 70 ml of Hoagland's nutrient solution (Menary and Staden, 1976) with no P.

The biostimulants UM, UR, MP and KGB are intended for application in agricultural contexts according to the manufacturer (The Glenside Group, Livingston, UK), whereas the SMFT,

SMMT and SMGS products have been developed with the aim of improving turf root zones according to the manufacturer (Symbio, Surrey, UK). Both sets of products intend to aid the development of soil health and biological diversity, with the objective being improved drought tolerance, growth rates and salt tolerance in crops. Table 3.1a explains the biological composition, carrier and experimental acronyms of the biostimulants used, and Table 3.1b shows the principal elemental composition of the biostimulants, and Table 3.1c shows recommended application rates and to experimental application rates. Intellectual proprietary reasons prevent disclosure of the full composition.

Table 3.1a: Descriptions of composition and function of commercial biostimulants used in the study. All biostimulants were stored and applied in powder form. There were two controls: with/without P fertiliser addition, but no biostimulant (Control, Control-P).

Biostimulant	Acronym	Carrier	Biology
Biagro MP	MP	Soluble powder	*Trichoderma* sp*., Glomus interadices, G. mosseae, G. aggregatum G. etunicatum, Paenibacillus* sp*., Psuedomonas* sp*., Bacillus amyloliquefaciens, B. subtilus, B. lichenoformis, B. circulans, B. Megatererium, B. pumilus, B. coagulans*
Biagro Brassika	KGB	Soluble powder	*B. amyloliquefaciens, B. subtilus, B. lichenoformis, B. circulans, B. megatererium, B. pumilus, B. coagulans*
MycoForce Fertiliser Treatment	SMFT	Soluble powder	AMF mix, *Trichoderma* sp., *Bacillus* sp.
MycoForce Mycorrhizal Transplanter	SMMT	Soluble powder	Stated as a mix of ecto and endo mycorrhizal fungi,, *Trichoderma* sp. and *Bacillus* sp.
MycoForce Mycorrhizal Grass Seed Coat	SMGS	Soluble powder	AMF mix, *Trichoderma* sp., *Bacillus* sp.
Humostart rhizobium	UR	Micro-ground DAP plus zinc, suspended in mono-potassium phosphate and di-potassium phosphate with zeolite	Several phosphate-mobilising & N fixing genera of bacteria (listed as *Bacillus, Paenibacillus, Azotobacter, Azospirillum* and *Beijerinckia*)
Humostart-mycorrhizae	UM	Micro-ground DAP plus zinc with zeolite and AMF blended through	Unconfirmed but very probably contains *Trichoderma, Glomus interadices, G. mosseae, G. ag gregatum* and *G. etunicatum*

Controls used in this study			
Autoclaved Biagro MP	MP(S)	Soluble powder	Sterilised
Autoclaved MycoForce Mycorrhizal Transplanter	SMMT(S)	Soluble powder	Sterilised
Autoclaved Humostart-mycorrhizae	UM(S)	Micro-ground DAP plus zinc with zeolite and AMF blended through	Sterilised

Table 3.1b: Elemental composition (%) of selected biostimulants (Medac Ltd., 2019)

Biostimulant	C	H	N	NO_2^-	NO_3^-	PO_4^{3-}	SO_4^{2-}
MP	37.02	6.11	1.92	<0.10	0.25	2.10	0.19
KGB	35.19	5.71	2.15	<0.10	<0.10	2.58	0.15
UM(S)	0.24	4.46	10.29	<0.10	0.17	71.23	4.37
UR	0.19	4.65	10.40	<0.10	<0.10	72.45	5.28
UM	0.17	4.31	10.59	<0.10	0.25	75.49	4.69

Table 3.1c: Biostimulant recommended application rates and experimental application rates. Quantities applied were between 1 and 400 times the recommended application rate per hectare to account of the fact that the small volumes of the recommended application rates which would not have been practical for the surface area of growing pots. Autoclaved controls were applied at the same rates as live treatments.

Biostimulant	Recommended application rate (kg ha^{-1})	Experimental application rate (g)
SMFT	0.1	0.1
SMMT	0.1	0.1
SMGS	0.1	0.1
KGB	0.15	0.15
MP	0.15	0.15
UR	0.15	0.15
UM	0.15	0.15

Table 3.2a: Characterisation of substrate used in trial, Compost *WI13*, with sharp sand. Analysis completed by Bulrush Horticulture on 26/07/16. Units are mg l^{-1}, unless otherwise stated. To avoid confusion, all mentions of "soil" in this report refer to the substrate described here

Density	pH	Electrical conductivity	Nutrient								
			NH_4^+	NO_3^-	TON	K	P	SO_4^{3-}	Ca	Na	Cl
264 g l^{-1}	3.6	16 µS cm^{-1}	7.87	0.8	7	1.2	4.4	8.8	0.7	5.3	10.5

Table 3.2b: Characterisation of lime and sharp sand added to compost to attain RB209 recommended pH. Analysis completed by Bulrush Horticulture. Units are mg kg^{-1}, unless otherwise stated

K	PO_4^{3-}	NH_4^+	NO_3^-
0.06 meq %	4.4	7.87	5.22

3.3.2. Data collection and analysis

Images of the *L. perenne* plants were captured using a RGB imaging in the Photon Systems Instruments (PSI) PlantScreen™ high-throughput conveyor system on the small plant platform at the National Plant Phenomics Centre (Plas Gogerddan, Aberystwyth University, Wales). The PSI facility utilises a conveyor system to move the pot-grown plants to the imaging area. The image analysis and feature extraction involved processing original images captured by the system, firstly by applying fisheye correction to remove distortion caused by the PSI wide angle camera lens. Segmentation and total projected plant area were calculated by transforming original RGB images to HSI colour space (Hue, Saturation and Intensity colour model, which was used because it represents colour in a similar way to how colour is detected by human eyes). Fixed thresholds were used to identify background pixels and plant pixels, therefore creating a binary image of the plant. Regions with areas registering less than a predefined threshold were removed. The total plant area was then calculated as the total number of plant pixels. Using IMAQ Edge Detection VI, the plant edge was extracted, and the plant perimeter values could therefore be obtained by calculating the number of pixels in the plant edge. The bottom position of each extracted region was fixed at immediately above the top of the pot. The smallest convex hull which could contain each plant was detected using OpenCV, which calculated pixel counts in each case. The ratio of plant area to convex hull

area was used to calculate compactness scores. This method is based on the approach described in Fischer *et al*. (2016).

At the end of the experiment (35 days post-inoculation), the leaf shoots were cut 3 cm above the hypocotyl–root boundary, and were subsequently dried for 2 days at 60 °C to determine values for dry biomass, along with manual measurements of plant height. The roots were washed from the potting substrate under cold tap water, and after weighing, a subset from plants of each treatment was taken to measure the extent of AM fungal colonisation. This process consisted of immersing the samples in 10% KOH, followed by rinsing in cold tap water, then autoclaving (121 °C, 100 kPa, 90 minutes) in a 5% acetic acid solution with 1% dye consisting of various inks (Vierheilig *et al*., 2005). Root colonisation with AM fungi was examined microscopically to assess the extent of total root length colonisation, as well as for the presence of mycorrhizal structures. Fifty grams of representative (thoroughly homogenised) potting substrate from each pot was dried at 60 °C for 3 days. Dried leaf, root, and potting substrate samples were milled to fine powder using a Foss CT193 (Cyclotec) before further elemental analysis. P concentration in plant leaf and root tissues was obtained from 1g of a milled sample of plant shoots and roots. After milling, the samples were ashed in a 500 °C furnace for 12 hours. The ashes were then dissolved in 1 ml of concentrated (14 M) HCl and made up to 50 ml with deionised water. Plant tissue orthophosphate concentrations were then measured using the Olsen P method (Murphy & Riley, 1962). Soil from the experimental pots was tested for plant-available P using a colorimetry method (Murphy & Riley, 1962). The data presented here, collected daily from the PSI system image analysis, involved the conversion of pixel count data to plant area, width and perimeter measurements in mm, along with a compactness score.

L. perenne showed a tendency to droop when they had been left growing more than four weeks since their last non-destructive cut, which made plant height and plant area measurements less reliable. Some of the larger plants began to occupy the space of their neighbouring plants after this four-week period, and since images were converted to binary for pixel calculations, it was not possible to reliably calculate plant area after this point since overlapping plants could not be distinguished. In the three weeks following the non-destructive cut, variations in individual plant growth had stabilised, making this period between plants reaching optimum growth and before they became too large for reliable measurement the most useful period for presenting data. As such, the data presented herein are from week 3 of the trial. Although the PSI imaging system provides estimates of plant height, it cannot take into account this tendency of *L. perenne* to droop as it matures, and therefore the height of

each plant was measured manually when the plants were removed from the conveyor system (mean taken of the three longest leaves).

Measurements for chlorophyll fluorescence were taken 4 weeks after the non-destructive establishment cut was taken (8 weeks after sowing). Chlorophyll fluorescence measurements were obtained using a CF Imager (Technologica Ltd.). Live plants from each treatment were placed in the CF imager and leaves were secured horizontally with an opaque clip. Preceding measurements, the plants were subjected to a ten minute dark-adapted phase, ensuring all PSII centres in the leaves were opened and no non-photochemical quenching was occurring. A low intensity light was then applied (25 µmol m^{-2} s^{-1}), which was sufficient to generate a minimum measure of chlorophyll fluorescence (F_o). The variable F_o' was obtained immediately after switching off the light. A saturating pulse (6,563 µmol m^{-2} s^{-1}) was then applied to the dark-adapted leaves which obtained maximum values of fluorescence (F_m) by forcibly closing reaction centres. Intensities of light were applied at increasing increments up to 2,000 µmol m^{-2} s^{-1}, interspersed by saturating pulses over 50 second periods. Experimental ambient temperature was maintained constantly at 21°C. In this study, it is assumed that measurements relate only to PSII, as signals from PSI are not considered significant below 700 nm (Murchie and Lawson, 2013).

Fluorescence (F_v) was the difference between F_o and F_m. The maximum yield of PSII photochemistry was calculated as F_v/F_m, which is accepted as a robust estimation (Murchie and Lawson, 2013), and in non-stressed plants the value is consistent around 0.83. As such, measurements F_v/F_m taken after the ten minute dark-adapted phase were considered an appropriate method for quantifying stress responses in the leaves of the experimental crop. The steady-state level of fluorescence is denoted by the quantity F'. Periods of 15 – 20 minutes were allocated to achieve F' levels after the dark–adapted phase. The variable F_m' was recorded by applying a saturating pulse to close all photosystem reaction centres, and in so doing provided a maximum value for fluorescence in the light-adapted phase. Non-photochemical quenching during the light-adapted phase means that F_m' takes lower values than F_m in the dark-adapted phase.

From these measurements, three parameters were calculated. ϕPSII is the operating efficiency of PSII, which was determined by calculating $F_m'–F')/F_m'$. This quantity is also denoted by F_q'/F_m' in other studies. This quantity describes the fraction of absorbed light used in photochemistry in the light-harvesting complexes of the plant, allowing an estimation to be made as to the rate of electron transport in PSII. The maximum efficiency of PSII in the light-adapted phase is described by the parameter F_v'/F_m', which provides an accurate measure of photo-inhibition. Decreases in the value of F_v'/F_m' are a result of increases in non-

photochemical quenching. The parameter F_q'/F_v' describes the amount of photochemical quenching in PSII. This value allows an estimate of the open fraction of light reaction centres. The variable non-photochemical quenching (NPQ) refers to the heat dissipation rate constant for chlorophyll excitation energy, which removes excess excitation in the light-harvesting complexes of the leaves, and in so doing mitigates free radical formation (Murchie and Lawson, 2013).

Images of the photosynthetic parameters F_q'/F_m' (PSII operating efficiency), F_v'/F_m' (maximum projected photosystem II operating efficiency) and non-photochemical quenching (NPQ, the rate constant of heat loss from photosystem II) were calculated using FluorImager (Technologica Ltd.). The model used to predict the relationship between light intensity and the photosynthesis rates were derived from the method of Eilers and Peeters (1988), and are outlined in Equation 3.1.

Equation 3.1: The model used for light intensity and photosynthesis rate calculations

$$ETR = \frac{PAR}{a \cdot PAR^2 + b \cdot PAR + c}$$

$$\alpha = \frac{1}{c}$$

$$ETR_{max} = \frac{1}{b + 2 \cdot \sqrt{a \cdot c}}$$

$$I_K = \frac{c}{b + 2 \cdot \sqrt{a \cdot c}}$$

ETR: relative rate of electron transport; PAR: photosynthetically active radiation.

Data were rendered in Excel and plotted using the R Studio package. Polynomial regression curves were calculated and tested for best fit using the predictor function to assign the appropriate polynomial order.

3.3.3. Statistical analysis

The data were summarised using Excel and the R Studio package, and were visualised using dplyr and ggplot2 packages. Local regression curves were plotted for the measure of each variable each day, and the ggfortify package was used to determine which statistical model would be most suitable to analyse each variable. To test the effect of replications, time period and biostimulant treatment, one-way analyses of variance (ANOVA) were carried out with subsequent post-hoc pairwise comparisons using Tukey Honest Significant Difference (HSD), applied at 95 % confidence level. Statistical analyses of light response curves consisted of Two-sample Kolmogorov-Smirnov tests between the Control+P treatment and the other biostimulant treatments across the interpretive variables described above. Frechet distance

between polynomial trajectories was also computed (Alt and Godau, 1995). Other methods used for comparing light response curves include fitting a double exponential decay function with zero-offset, and testing significance using the Wald test (Rascher *et al*, 2000).

PSI generated image analysis data was carried out for the three-week period (comparisons between week 1 and week 3 are shown in Fig 3.2), and results are presented in the form of boxplots displaying data for each morphometric variable during the final experimental week. Boxes show extent of upper/lower quartiles around the mean, and whiskers show upper and lower extent of data. Asterisks denote levels of significance. P-values below 0.05 are flagged with one asterisk (*), p-values below 0.01 are flagged with two asterisks (**), p-values below 0.001 are flagged with three asterisks (***), and p-values below 0.0001 are flagged with four asterisks (****). Grey circles denote extreme outliers (Figures 3.2-3.8).

3.4. Results

There were significant increases observed in the dry biomass of plants treated with the biostimulants UM, UR and UM(S) compared with the controls when measured at the end of the experiment (Figure 3.2, ANOVA, p-values denoted by asterisks). Biomass from harvested plants of all other treatments and controls were relatively consistent and showed no significant differences, and none weighing more than 0.05 g DM. Overall mean plant area over the duration of the experiment in treatments UM(S), SMMT and UM was significantly greater than the control (ANOVA, $p < 0.001$). For the first seven days, there were no significant differences between the treated replicates and controls (ANOVA, $p < 0.05$). By the second week, there were significant increases in mean plant area in three treatments, UM(S), SMMT and UM when compared with the controls (ANOVA, $p < 0.0001$). By the final week, there were significant differences between mean plant area in six treatments, with four treatments resulting in significantly greater plant area than the controls, these being UM(S), SMMT and UM (Figure 3.3, ANOVA, p-values denoted by asterisks). Two treatments, SMFT and MP, had a significant decrease in plant area compared with the controls. The increased plant area and biomass from plants treated with the UM and UR biostimulants is not unexpected, as their carrier substrate consists of over 70% P by mass (Table 3.1b), and the potting substrate was P-limited. Both the live and sterilised UM treatments were associated with slightly higher biomass than UR treated plants, despite having carrier substrates of identical chemical composition, which could in theory be suggestive of some microbially-mediated benefits. The uncertainty was also greater across plants treated with the sterilised UM treatment. Growth curves for plant area across all treatments showed that the UM(S) treatments reached the highest maximum area (~ 3,000 mm^2 mean leaf area), and unlike all other treatments, growth curves of UM(S) did not appear to plateau as quickly (Figure 3.3).

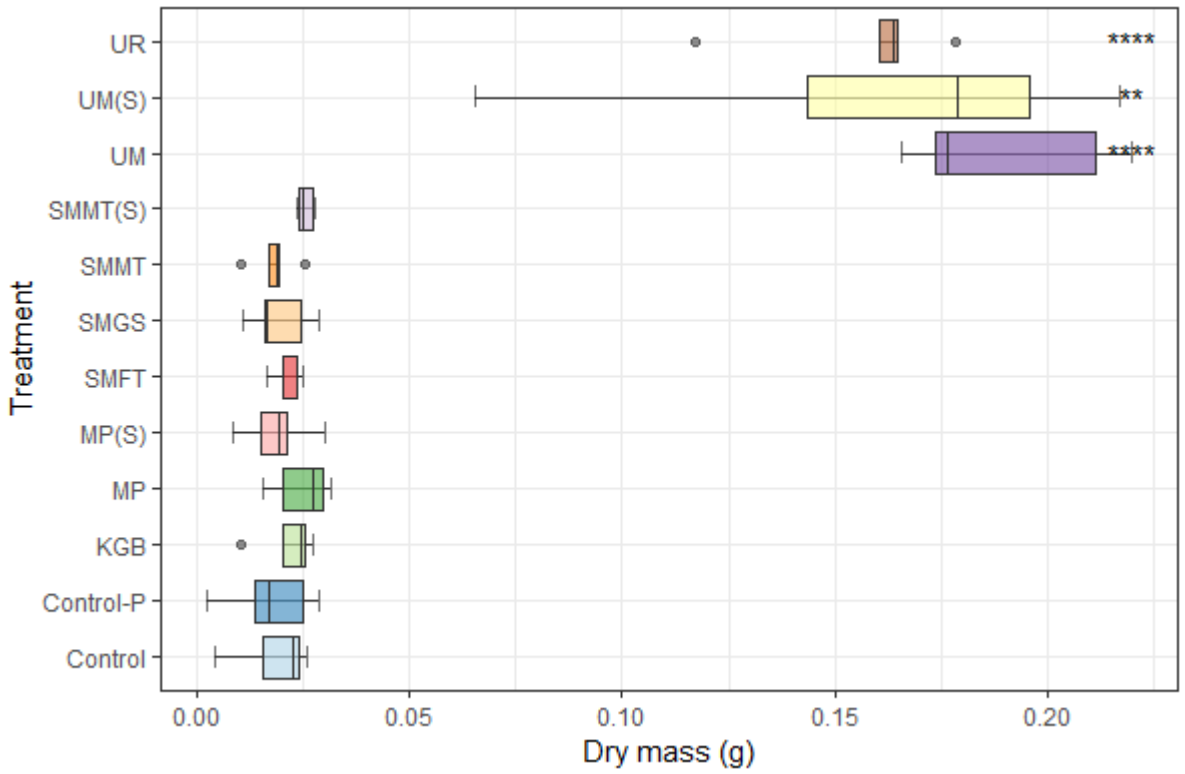


Figure 3.1: Boxplot summary of mean plant dry biomass (g) attained for each biostimulant treatment (*n* = 5)

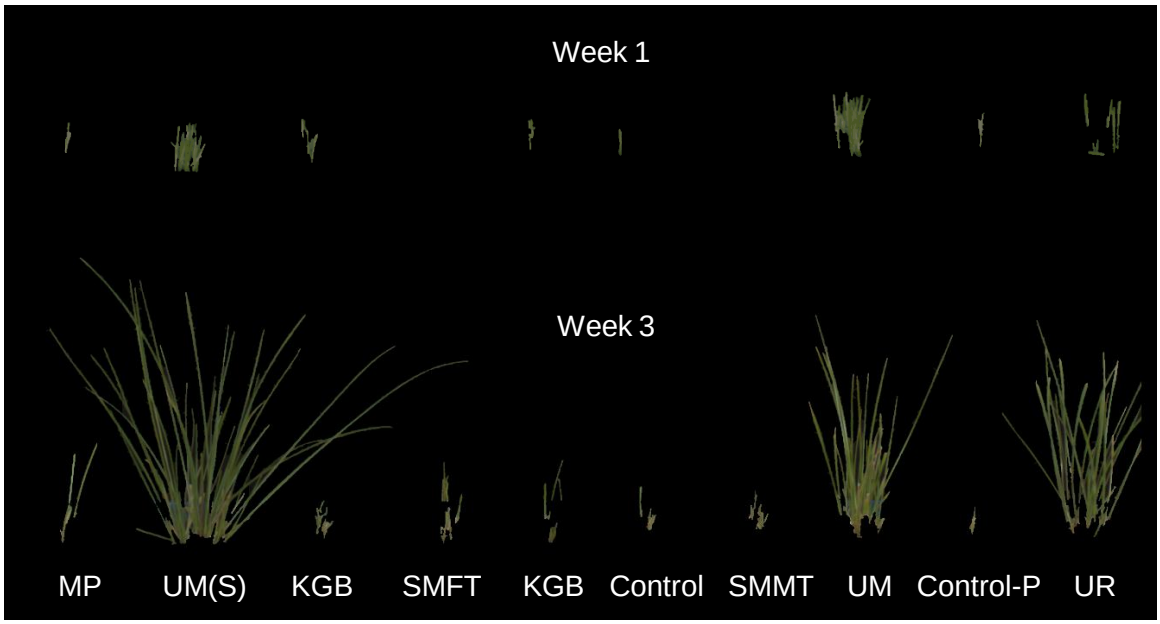


Figure 3.2: Comparisons of the same *Lolium* plants between week 1 and week 3 demonstrating the effects of biostimulant treatment. This graphic is comprised of masked RGB images which have undergone fisheye correction. The advantages of measuring plant area and perimeter as opposed to height and width are evident here, with some evidence of tillering in three specimens and the tendency of the plants to droop in the third week.

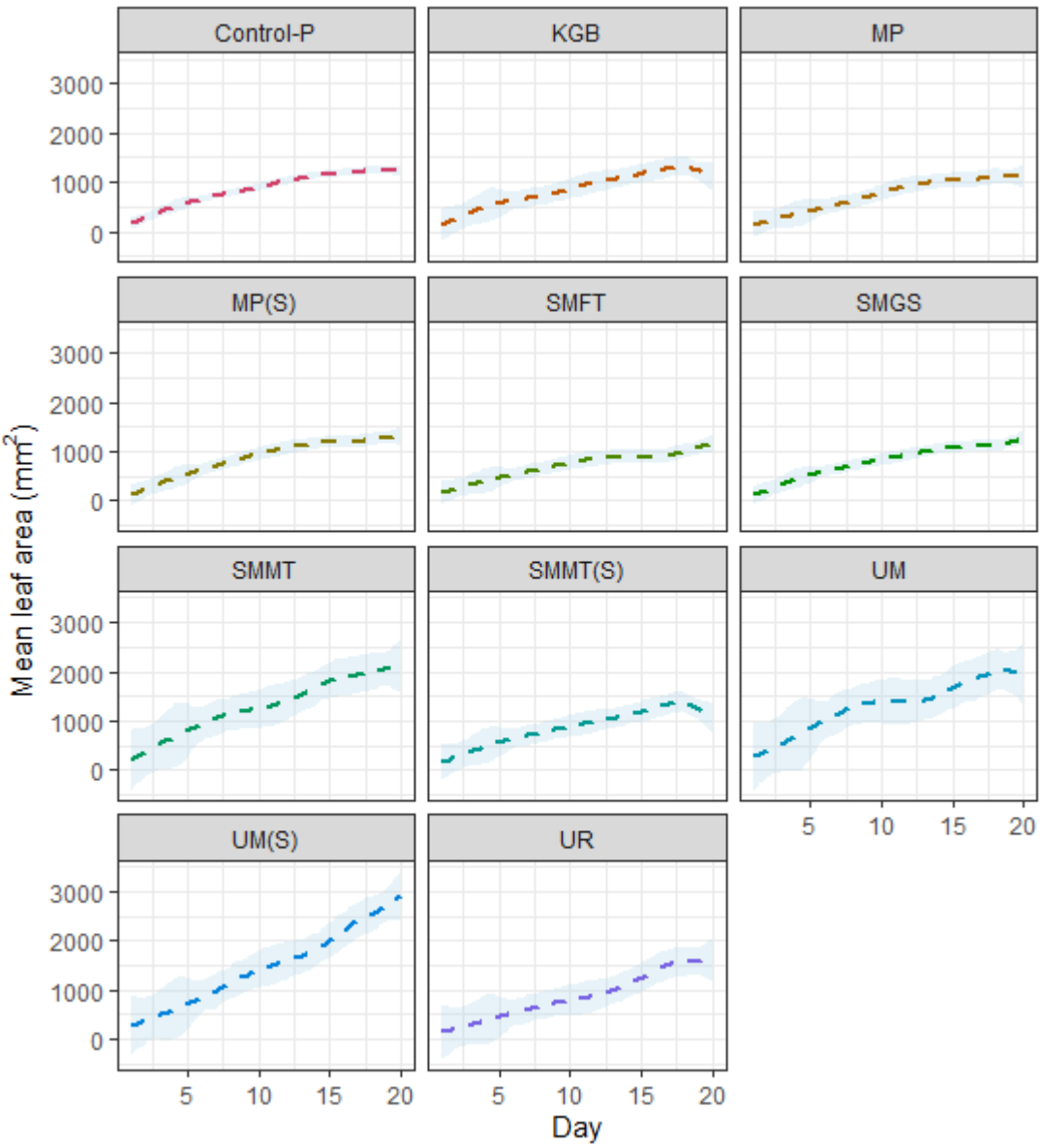


Figure 3.3. Normalised plant area curves. Shoot areas for plants receiving each biostimulant treatment as extracted from RGB side images of the plants. Curves denote daily mean; shaded areas denote +/- SE, 95 % confidence interval (n = 5 plants)

Compactness is the ratio between plant area and the area of the convex hull which can be drawn around the plant. This parameter describes the relationship between petiole length and leaf blade width. For the first seven days, there were no significant differences between the replicates and the controls (ANOVA, $p < 0.05$). In the second week, a post-hoc test showed significant differences between UM and six other treatments (ANOVA, $p < 0.001$). By the final week (Figure 3.4a), there were significant differences between eight treatments and the control, with three treatments resulting in a significantly higher compactness ratio than the control, these being UM(S), SMMT and UM (ANOVA, $p < 0.01$). Five treatments, Control-P, SMMT(S), MP, SMGS and SMFT all had a significantly lower mean compactness ratio than the control treatments (Figure 3.4a, ANOVA, p-values denoted by asterisks). A significant increase in overall plant perimeter between treatments was observed in UM(S) over the duration of the experiment (ANOVA, $p = 0.02$). For the first seven days, there were no significant differences between the replications (ANOVA, $p > 0.05$). In the second week, there were significant differences between two treatments (UM(S) and SMMT, ANOVA, p-values below 0.001). By the final week, there were significant differences between seven treatments and the control, with five treatments resulting in a significantly higher compactness ratio than the control, these being UM(S), SMMT, UR, UM(S) and UM (Figure 3.4b, ANOVA, p-values denoted by asterisks). Two treatments, MP and SMFT, had significantly lower mean plant perimeters than the control treatments. A significant increase in overall plant width was observed in plants treated with UM(S) compared to the controls over the duration of the experiment (ANOVA, $p = 0.03$). For the first seven days, one treatment (MP) had a significantly lower width than the other treatments (ANOVA, $p < 0.001$). In the second week, no significant differences between plant widths were observed across replicates (ANOVA). In the final week (Figure 3.4c), a post-hoc test (TukeyHSD) was able to discern significant differences between ten treatments and the control, all of which produced significantly greater mean plant widths. These treatments were UM(S), Control-P, UM, UR, SMGS, SMMT, KGB, MP, MP(S) and SMMT(S) (Figure 3.4c, ANOVA, p-values denoted by asterisks). When measured at the end of the experiment, there were significant increases observed in the height of plants treated with the biostimulants KGB, UM, UR and UM(S) compared with the controls (Figure 3.4d, ANOVA, p-values denoted by asterisks).

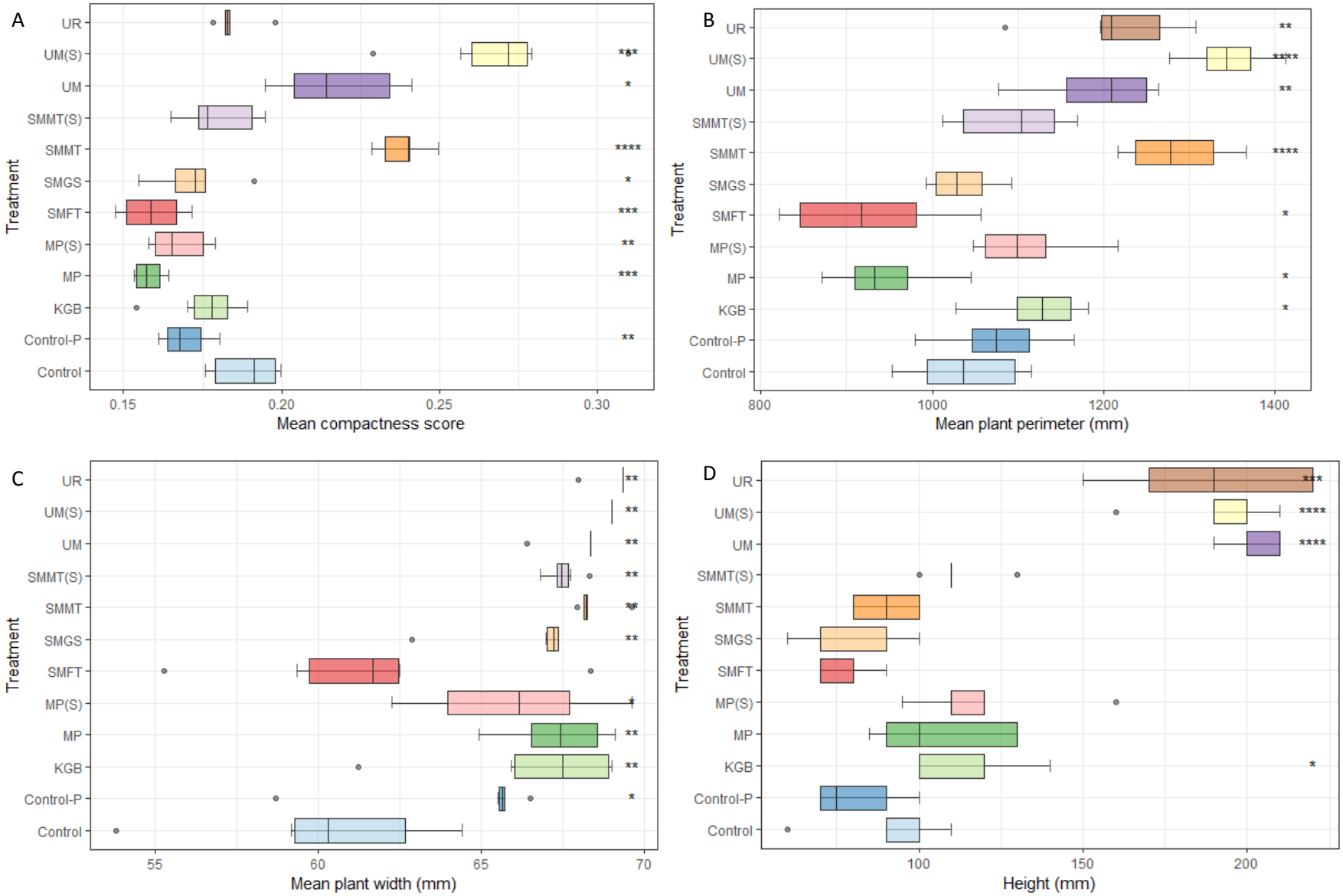


Figure 3.4: Boxplot summaries of mean plant compactness (A), perimeter (B), width (C) and height (D) for each biostimulant treatment in week 3 (n = 5)

Water use efficiency in three treatments (UM, UR and UM(S)) was significantly higher than other treatments (Figure 3.5). Interestingly, other biostimulant treatments also appeared to have slightly lower water use efficiency than the control treatment. This may be evidence of a possible metabolic cost to the plant from forming a symbiosis that did not translate into a tangible yield or health benefit. The UR treatment also had slightly lower water use efficiency than the UM treatments across the experimental period, following a trend observed across a number of parameters. A possible explanation for this could be that in the experimental conditions applied in this experiment, PGPR were less beneficial to the plant than AMF symbioses, but this cannot be robustly demonstrated without qPCR data showing extent of root colonisation in UM (mycorrhizal) treated plant in comparison to UR treatments (Řezáčová *et al*., 2018). Although a root staining method was followed, the results were inconclusive, which was likely due to an ineffective dye being used.

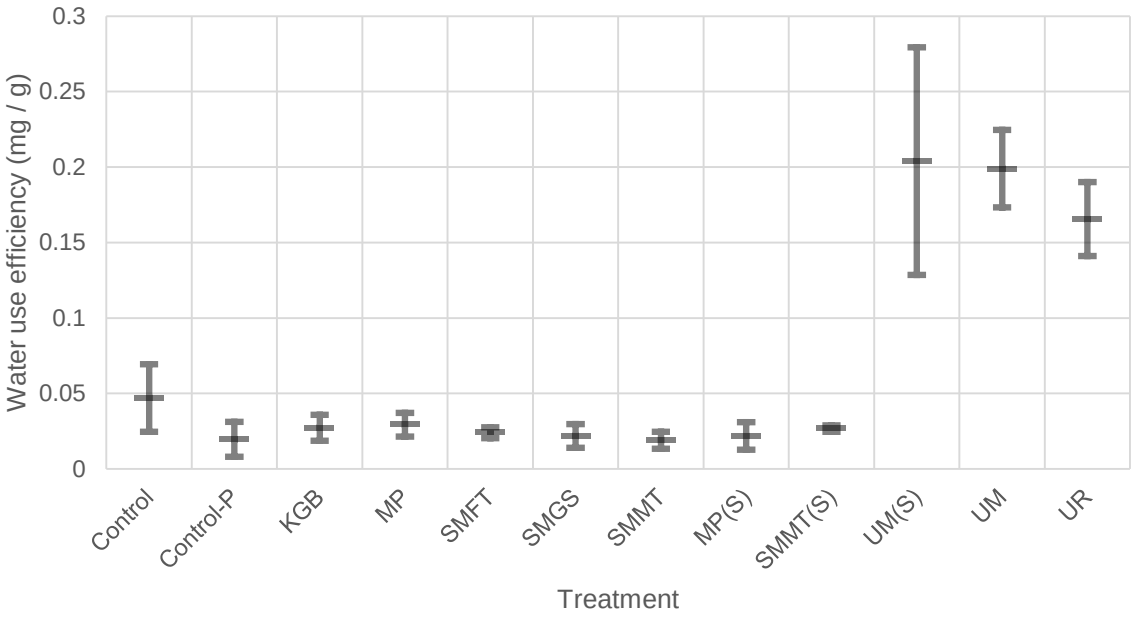


Figure 3.5: Summary of water use efficiency (mg / g water), estimated gravimetrically by dividing dry biomass at the end of the experiment by the total amount of water added to plants under each biostimulant treatment (n = 5). Central line denotes mean, error bars show standard deviations.

3.4.1. Plant-available phosphorus

There were significant increases observed in the plant-available P in soil treated with the biostimulants UM, UR and UM(S) compared with the controls when measured at the end of the experiment (Figure 3.6a, ANOVA, p-values denoted by asterisks). Soil P concentrations of the other treatments were fairly similar, with the exception of the Control-P treatment, which was considerably (although not significantly) lower. These results are fairly close to what was expected, in that the Control-P treatment did not receive RB209 levels of P and was therefore severely P limited. The P levels of the UM, UR and UM(S) treatment were expected to be higher, as the carrier substrates contained over 70% PO_4^{3-} by elemental composition. The soil P levels of these treatments is in the region of four times higher than the other treatments, and since application rates were over 50% higher than the RB209 application rate, and the form of P fertiliser was 47% plant available P, these results suggest very little P has been leached from the potting substrate. There were also significant increases of P in plant root and shoot tissues in plants treated with the biostimulants UM and UM(S) compared with the controls (Figure 3.6b ANOVA, $p < 0.05$), as well as significantly lower P in the root and shoot tissues of the P-limited control P plants (ANOVA, $p < 0.01$). The reduced P in the P-limited control plants is not unexpected for the same reason as above, but it is interesting that the root and shoot tissue P concentrations in the UR treated plants were not significantly different to the control, but were very similar to P-levels in SMFT treated plants, despite having much higher plant available soil P. Overall, the root and shoot tissue P levels across treatments were considerably less divergent than the plant available soil P levels. P tissue levels were in the order of a quarter of soil P levels, which is broadly consistent with expectations, as plants were not expected to take up all soil P, but also the extractants were different between methods (acetic acid for soil and hydrochloric for plant tissue extractions), and some discrepancy was expected. No biostimulant treatment was associated with significantly lower soil or plant tissue P. Some of the morphometric variables measured in this study have been suggestive of a metabolic cost of a symbiosis mediated via inoculation, one instance being the SMFT treated plants having significantly lower plant widths and perimeters than the control treatments, but comparable plant tissue P levels to the UR treated plants. Interestingly, whatever process responsible for the reduced growth in SMFT treated plants is not associated with reduced leaf tissue P, which from this data is comparable to plant tissue P levels in UR treated plants.

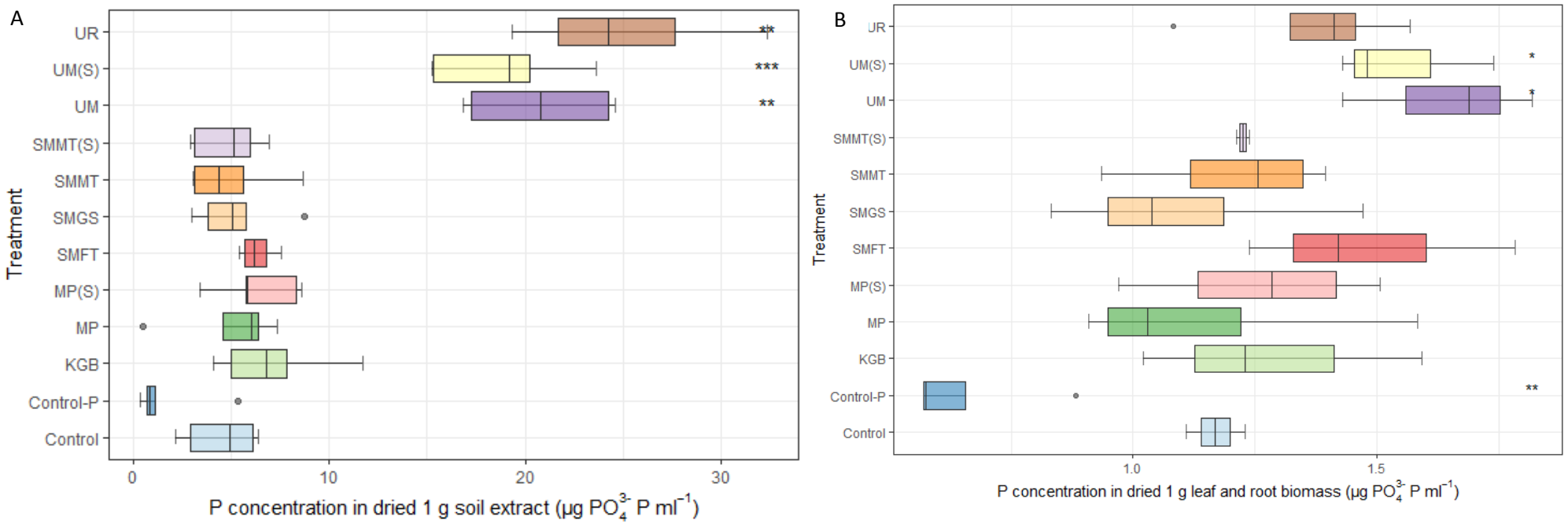


Figure 3.6: Mean PO_4^{3-} (plant-available phosphorus) concentrations obtained from GEN5™ microplate analysis of each biostimulant treatment in in 1 g dried soil extracts (A) and 1 g dried leaf and root biomass extracts (B) (n = 5)

3.4.2. Chlorophyll fluorescence

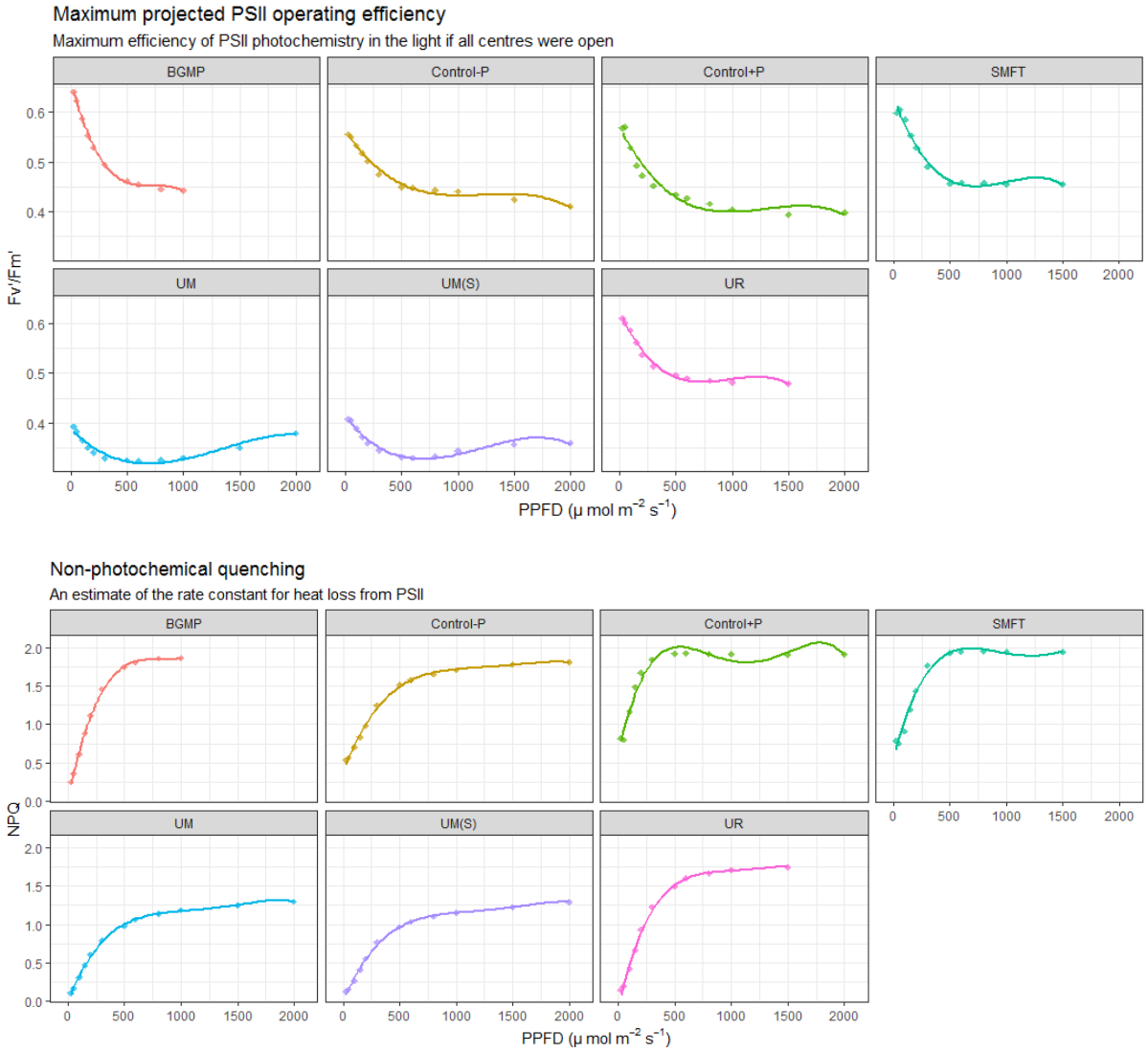
Chlorophyll fluorescence measurements were taken from plants undergoing five biostimulant treatments and two controls, with the tests described in the methods section repeated on different plants undergoing the same treatments obtaining similar results. Two-sample Kolmogorov-Smirnov tests were carried out between treatments with Control+P as the reference group, with results summarised in Table 3.3. ϕPSII provided a measure of overall photosystem II efficiency, and no significant differences were observed between polynomials fitted to any biostimulant response and the P-amended control treatment.

Table 3.3: Summary of P values between Two-sample Kolmogorov-Smirnov tests carried out between all biostimulant treatments and the Control+P treatment across the four key variables used to interpret chlorophyll measurements. Asterisks denote levels of significance. P-values below 0.05 are flagged with one asterisk (*), p-values below 0.01 are flagged with two asterisks (**), p-values below 0.001 are flagged with three asterisks (***)

Treatment	F_v'/F_m'	F_q'/F_v'	NPQ
UR	***0.02 ****	***0.04 ****	***0.04 ****
UM	***3.34E-05 *****	0.10	***0.002 *****
UM(S)	***0.001 *****	0.10	***0.002 *****
BGMP	0.21	0.13	0.13
SMFT	0.08	0.99	0.19
Control-P	0.48	0.25	***0.03 ****

Maximum projected photosystem II operating efficiency (F_v'/F_m') was calculated (Figure 3.7) and some significant differences between treatments were observed (Table 3.1). UR, UM and the UM sterilised control all had significantly lower maximum projected PSII operating efficiencies than the controls. Maximum photosystem II efficiency to operating efficiency (F_q'/F_v') was calculated, and the UR treatment was found to have a significantly lower curve than the P- amended control (Table 3.3). UR curve function continued to deteriorate below 0.5 under intensities of 1,500 µmol m^{-2} s^{-1}, whereas the P-amended control plateaued above 0.4. Non-photochemical quenching ($F_m – F_m'$)/(F_m') curves were calculated (Figures 3.7 and 3.8), and significant differences between the P-amended control and biostimulant treatments were observed (Table 3.3). The non P-amended control, UR, UM and UM(S) treatments were all associated with significantly lower rates of non-photochemical quenching than the P-amended control. Maximum projected photosystem II operating efficiency was significantly higher than the controls in the plants treated with two biostimulants consisting of mycorrhizal fungi and

rhizobacteria, biofixed to a zeolite-DAP carrier (UR, UM and UM(S)). Non-photochemical quenching in the plants treated with these biostimulants was also significantly lower, suggesting that in this study a linear relationship existed between the two variables. These differences are associated with biostimulant treatments which yielded higher biomass and had higher water use efficiency.



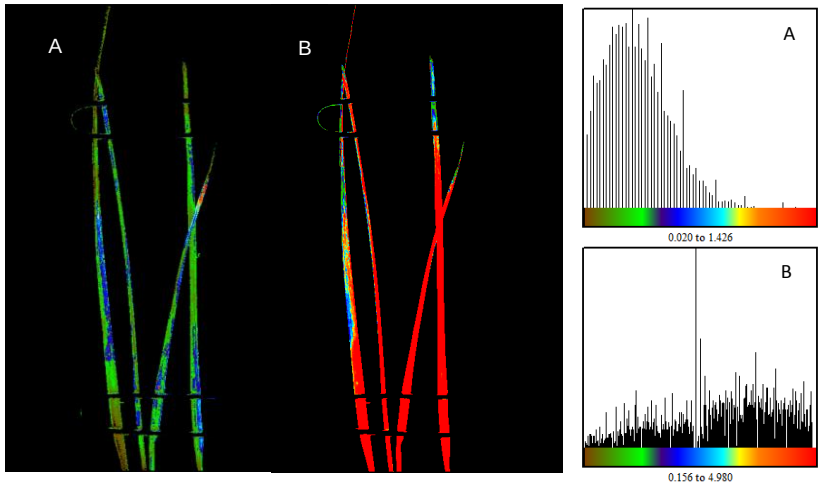


Figure 3.8: Non-photochemical quenching in grasses treated with UR biostimulants and corresponding histograms depicting NPQ range across leaf surface area (**A**) after a the dark-adapted phase followed by a saturating pulse and (**B**) after incremental rises in light intensities interspersed with saturating pulses up to a maximum intensity of 1,500 µ mol m^{-2} s^{-1}. Resolution = 200 µm

Figure 3.7: Maximum projected photosystem II operating efficiency (F_v'/F_m', fitted curves represent third order polynomials) and non-photochemical quenching ($(F_m – F_m')/(F_m')$, fitted curves represent fourth order polynomials) describing the rate constant of heat loss from photosystem II as measured in plants across all biostimulant treatments (n = 5).

3.5. Discussion

In this study, data from harvested biomass and plant height analysis for plant-available P suggest that three biostimulant treatments produced some significant growth advantages in *L. perenne*, these treatments being UM(S), UM and UR. The colourimetry method for testing plant-available P was able to discern clear differences between soils taken from these three treatments, which all had significantly higher plant-available P than the controls and competing biostimulants. Root and leaf P levels were also increased by these three treatments (although only significantly so by UM and UM(S)). The evidence these tests produced suggests that much of the plant growth advantages seen in these treatments can be explained by increased plant-available P (Hammond *et al*. 2009). The biostimulant carrier composition in the UM(S), UM and UR treatments contained >70 % phosphorus, which could account for much of the improved plant growth in these treatments, as in the KGB and MP treatments the plant-available P fraction present in the carrier was less than 3 %.

Although this study measured root tissue P, which has allowed some conclusions to be made about crop P uptake from the biostimulant treatments, it would have also been useful to have measured root length and morphology, which can now be done by root scanners, which could have provided information about root extent, branching and surface area (Poorter *et al*., 2012; Hammond *et al*., 2009). One of the purported benefits of AMF biostimulants frequently mentioned in both literature and commercial contexts is that inoculation improves root structures, either directly or indirectly via improved soil aggregation. Furthermore, it would have been useful in this study to have utilised other methods of measuring AMF colonisation levels in the treated plants. The extent and length of AMF structures could probably have been detected using a better root staining dye, e.g. Trypan Blue B (Vierheilig *et al*., 2005). If qPCR had been used in this method, it would have been possible determine not just the species of AMF in the roots but the absolute abundances per unit weight of roots (Řezáčová *et al*., 2018).

This study, while not subjecting crops to an abiotic stressor, has nevertheless examined two proxies that pertain to the ability of the experimental crop to access soil water and use it efficiently, which is very much linked to the drought tolerance of a crop (Munns *et al*., 2010). These proxies were crop water use efficiency and chlorophyll fluorescence. By using light response curves to measure chlorophyll fluorescence, it can be possible to observe signs of stress in plants much earlier than using traditional methods (Petrozza *et al*. 2014), although in this study the crop growing period was too short for such observations to be made. The other was water use efficiency, which indicates how much biomass plants can produce per unit water supplied (Chen *et al*., 2011). As such, plants with increased water use efficiency have

potential for improved yield under drought stress (Honsdorf *et al*., 2014; Chen *et al*., 2011). Since this study suggested that there may have been differences in photosynthetic and water use efficiency between plants treated with different biostimulants, it would have been very informative to have incorporated an abiotic stress variable into the experimental design, ideally drought or salt stress (Forni *et al*., 2017). This would have allowed comment on other, interactive effects with the other experimental factors, particularly with the P availability (Hammond *et al*., 2009), which would have been particularly valuable in this study given that commercial biostimulants are usually purported to offer increased abiotic stress resilience (Calvo *et al*., 2014).

Some negative effects of colonisation may account for the water use efficiency and plant width to have been lower in some biostimulant treatments compared to the control treatment, which could be due to a negative metabolic cost of a symbiosis that has failed to translate into a yield increase. Similar studies have observed similar behaviour in experimental crops, for example Řezáčová *et al*. (2018), who report significantly reduced aboveground respiration when roots of an experimental grass crop were colonized by AMF, as well as decreased photosynthetic efficiency, particularly at low P levels. It would have been useful to have measured respiration in this study to observe treatment effects, especially if C allocation or C fluxes from roots could also have been studied to better understand implications of water use efficiency on carbon allocation and storage. In this study, some of the biostimulant treatments resulted in significantly lower morphometric measurements than the control treatments, such as SMFT with respect to plant width and plant perimeter, and a possible explanation presented by this study has been that there has been a metabolic cost to the plant as a result of a symbiosis that is draining the plant of carbon. Since no plant tissue P levels in biostimulant treatments were lower than the controls, this possible metabolic cost remains only conjecture based on this data. Measuring C fluxes around plant roots would be one method of properly examining this, as carbohydrate import and export could then be ascertained (Fatichi *et al*., 2014; Martínez-Vilalta *et al*., 2016), which would be the probable drain on the plant's resources if a metabolic cost of inoculation were in action.

Ideally, photosynthetic efficiency would be continually measured between treatments at different P levels as opposed to at a single time point, as this would offer much clearer insights as to whether biostimulant treatment upregulated photosynethsis. Řezáčová *et al*. (2018) propose that AMF symbiosis does upregulate photosynthesis either by improved mineral nutrition or C sink stimulation. Scanning the experimental crop with infrared light can provide data on leaf temperature, and near infrared imaging can offer information on plant water content (Honsdorf *et al*., 2014). Such data collection methods in combination with a drought or salt stress variable could allow meaningful conclusions to be made as to whether the

biostimulant treatments that have been associated with improved mineral nutrition in this study would also offer any of the purported health benefits to the crop, and infrared imaging could be easily incorporated into a similar experimental design to this study. A useful combined approach would be to use multi-spectrum analysis (infra-red, visible and ultraviolet light) of reflected or re-emitted light, which will provide data on plant health status, hydrological efficiency and tissue composition, which has been used to great effect by Petrozza *et al*. (2014) to demonstrate some positive plant health benefits associated with biostimulants. Their study also subjected crops to a genuine stress response as well as measuring the relevant proxies, which allowed conclusions to be made about biostimulant efficacy with some robustness. Their study examined an experimental tomato crop, and in Europe, tomatoes are grown commercially in controlled environment glasshouses, meaning that in their case differences between glasshouse and field conditions were not economically relevant caveats. The same cannot be said of the experimental grass crop grown in the present study, and therefore much greater caution is needed when interpreting these results and making conclusions about the relevance of the study to field conditions. This is because field conditions involve many biotic and abiotic variables which were not present in this study which govern establishment and efficacy of biostimulants (Hart *et al*., 2018; Kaminsky *et al*., 2019). The biotic variables include native microbial communities in soil and metabolic niche overlap, and the abiotic variables include pH, temperature, salinity, compaction, rainfall and atmostpheric nitrogen deposition. A biostimulant will not be efficacious if the plant growth-promoting microbial fraction introduced cannot compete against native microbial communities for the same metabolic niche. As such, all of the conclusions made from the data in this study are purely indicative as far as they may have relevance to agricultural contexts, and require further investigation in field experiments.

In this study, there should have been sufficient N for the experimental crop because Hoaglands solution was added in the first two weeks, but it would still have been useful to have measured soil and leaf N to see whether any of the treatments affected N in the plant tissues. Although the microplate assay of plant-available soil P suggested that the main explanation for enhanced plant tissue P and plant growth was increased plant-available P, there may also be indirect pathways for microbial biomass to release nutrients (Hammond *et al*., 2009; Marschner, 2011). In similar fashion to carrier substrate phosphorus, the manufacturers of the biostimulants specify that zeolite is present in all the biostimulants tested in this study except MP and KGB. In the case of the Symbio products, the microbes are “biofixed” to the zeolite, which the manufacturer claims allows survival of the microbes for at least one growing season (Symbio, 2020). It is likely that a number of factors will determine whether this is the case, such as whether the microbes are able to form biofilms, their inherent

resistance to anti-bacterial and anti-fungal substances and how competitive the microbial community of the soil into which the biostimulant is applied. The use of the naturally occurring alumino-silicate mineral zeolite in agriculture has been well-documented as a method of improving nutrient use efficiency of organic fertilisers on account of their high cation exchange capacity (Ahmed *et al*, 2010; Ramesh and Reddy, 2011). This is a function of the large internal surface area in their structure which is associated with reduced nitrification and N-leaching.

Whether the activity from plant growth-promoting microorganisms derived from or stimulated by the biostimulants contributed to the plant growth cannot be confirmed or discounted based on the data in this study, which did not include qPCR or equivalent methodology that could have offered such insights (Řezáčová *et al*., 2018). The sterilised treatment UM(S) produced statistically similar results to the UM and UR (non-autoclaved) treatments, suggesting that nutrient effects may have greater influence over *L. perenne* growth in the described conditions than microbial effects. However, the non-autoclaved UM and UR treatments did produce more consistent results in all variables than the UM(S) (autoclaved) treatment, and had fewer outliers. All data collection methods recorded that the autoclaved biostimulant UM(S) treatment produced growth results comparable to, and in some cases exceeding that, of its non-sterilised counterpart (UM). This suggests that the microorganisms present within the UM biostimulant did not necessarily contribute to the enhanced growth observed on plants treated with it, and possibly there were metabolic costs to the AMF symbiosis that drained the plants of carbohydrates without offering increased P nutrition, although without knowing the extent of colonisation this cannot be verified. It may still be the case that microbial activity contributed to plant growth-promotion as a result of the treatment. Biostimulant treatments may have stimulated microbial biomass growth on account of improved nutrient availability, but microorganisms would not have been introduced directly to the soil by the treatment. If this is the case, the treatment could not fall under the European Biostimulants Industry Council definition, which is “a material that contains… microorganisms whose function…is to stimulate natural processes to benefit nutrient uptake… and crop quality” (EBIC, 2019). It would be possible to determine whether fungal biomass in the rhizosphere of plants treated with sterilised biostimulants was increased by carrying out an ergosterol assay (Weete *et al*., 2010), although this method does not identify AMF. AMF are by no means the only plant growth-promoting fungal phylum, but they are the fungal taxa claimed by the manufacturers to be included in the biostimulant formulation. Next Generation Sequencing techniques could suggest changes in fungal community structure associated with biostimulant inoculation (Detheridge *et al*., 2016), which could be combined with qPCR to determine the extent of colonisation (Řezáčová *et al*., 2018). This method could then quantify the extent to which biostimulants actually introduce microbial species associated with plant growth promotion.

However, such a method in a trial using compost as an experimental substrate would not be able to account for properties of agricultural soil that are now known to influence biostimulant efficacy, such as priority effects and metabolic niche overlap.

The PSI system was able to provide generally useful information which corresponded reasonably with manual measurements, and the PSI automated watering component ensured that differences in water availability could be discounted as a source of variation. There was some discrepancy between the manual measurements and the PSI system data. One such case was with the treatment SMMT, which was reported from PSI data to have significantly higher plant area, height and perimeter than the controls, but manual height measurements, biomass measurements and visual assessments contradicted this. One possible reason for the discrepancy in height data is that despite the PSI imaging system providing estimates of plant height, it is unable to take into account the tendency of *L. perenne* to droop as it matures. In general, 2D imaging has several shortcomings which affects the accuracy of the information it can produce in the context of leaf growth data. 2D imaging cannot collect as much spatial data as 3D imaging, and is not capable of analysing volumetric data at all. It is now possible to implement 3D mesh algorithms upon image compression, which could increase the reliability of the morphological data estimation. This is not as effective, however, as full scale 3D imaging (Duan *et al*., 2018). There do exist several 3D plant imaging software platforms (such as RootReader3D) which are able to estimate plant root volumetric data. However, there does exist a trade-off between the accuracy and detail 3D imaging can afford as opposed to the efficiency of 2D imaging, and its suitability for high-throughput analysis.

In this study, the experimental method could have benefited from using autoclaved controls of all the biostimulants, and analysing soil both before and after the plant growth was measured, and testing the availability of plant-accessible P level when biostimulants were applied to composts without plants growing in them. This would have allowed observations on how biostimulants may affect soils before crops are planted. The issue of PSI image uncertainty could be addressed by restricting lateral growth of plant leaves, or by trialling a crop species which could be measured by an overhead camera (and therefore measuring a leaf rosette (Santaniello *et al*., 2012)).

The relationship between PSII electron transport efficiency and improved carbohydrate import in the plants treated with UR, UM and UM(S) biostimulants may be a function of the fact that the products of electron transport (ATP and NADPH) are directly used in carbon assimilation (Murchie and Lawson, 2013). Improved P availability in the tissues of plants treated with these biostimulants may be associated with improved photosynthetic efficiency by ensuring that

plant cells are not P-limited, and as such, ATP concentrations are not a limiting factor in carbohydrate import (Hammond *et al*., 2009).

One of the fungal genera present in the UM treatments was *Trichoderma*, which are linked to directly improved abiotic stress tolerance in host plants (Mukherjee *et al*., 2013). The measure of NPQ from stress light responses can be used to estimate a plant's resilience to abiotic stress, and in this study, the UM treatment produced significantly lower NPQ curves than the control, which may indicate an improved physiological plasticity in the leaves of the UM treated plants. Along with the improved water use efficiency observed in plants treated with this biostimulant, both of these measurements could be suggestive proxies of improved physiological plasticity in the experimental crop (Großkinsky *et al*., 2015), but in the absence of an abiotic stress variable in the experimental design, this remains conjecture, and requires a repeat of the experiment with stress factors included in the method (Forni *et al*., 2017).

In any case, the sterilised UM treatment produced highly similar light response curves to the live treatment, which suggests that mineral effects are likely to have contributed to these results, and any effects from beneficial microorganisms in the soil cannot have been introduced directly via biofixed microbes associated with the live biostimulant. This does not necessarily mean that plant growth-promoting microorganisms did not contribute to improved resilience to abiotic stress, but if such organisms experienced improved rhizosphere equitability post-inoculation of the biostimulant, then it must be associated with the chemistry of the zeolite-DAP carrier substrate (Ahmed *et al*., 2010). The biostimulants treatments associated with the lowest NPQ rate constants in this study consisted of a DAP and zeolite carrier, and no differences were observed between the live and sterilised forms in response curves. This suggests that the lower NPQ curves may be associated with improved mineral use efficiency, but whether the microbial composition of the biostimulant had any significant role in this remains unclear. Further research is necessary to quantify shifts in rhizosphere microbial community structure post-inoculation with biostimulants in order to identify whether increased abundance of taxa associated with plant growth-promotion are in fact associated with these treatments.

In this study, conclusions have been based on the assumption that the relationship between F_q'/F_m' and CO_2 assimilation rate was linear. This is because unlike chlorophyll fluorescence field measurements, the experimental conditions in this study were tightly controlled, with ambient temperature constantly maintained, a consistent dark adapted phase and a carefully controlled growing environment maintained to ensure plants were not subject to abiotic stressors. Murchie and Lawson (2013) describe in detail these factors which may affect the

robustness of linearity assumptions, which mainly relate to whether experimental conditions can be controlled.

3.7. Conclusion

The method used herein was able to effectively document differences in growth rates, morphology and crop P uptake of a grass crop treated with different biostimulants and phosphorus amendments. Improved availability of phosphate accounted for much of the differences observed between treatments and mineral nutrition of the crop. The similarity between behaviour of plants treated with sterilised and live forms of the same biostimulant suggests that mineral effects may have greater influence over *L. perenne* growth in the experimental conditions used in this study than microbial effects, but this does not take into account how biostimulant application affected the growing conditions, soil microbial diversity, resilience to drought or salinity of the treated plants. Future research must explore how biostimulants affect soil health and microbial diversity and must also discuss whether this has an impact on crop resistance to environmental stress factors.

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Chapter 4

A phenomics approach to measuring *Arabidopsis thaliana* response to biostimulants

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4.1. Abstract

Major increases in the use of chemical fertilisers in agriculture contributes significantly to global soil, air and water pollution. In tandem with increases in global food demand, precipitated by population growth, sustainable and integrated nutrient management regimes to safeguard food supplies are becoming increasingly necessary, and biostimulants are increasingly advocated as a component of future sustainable crop production. The present study examined the effect of three commercial biostimulants on an experimental *Arabidopsis thaliana* crop, using a conveyor system and plant imaging method was to measure daily plant growth and various morphology parameters relating to biomass accruement. Significant increases in growth rate were associated with mycorrhizal and rhizobium biostimulants, which consisted of DAP and a zeolite carrier. Some live biostimulants improved plant growth and morphology to greater effect than under in non-P amended treatments, but biostimulants were associated with lower leaf tissue P than the control treatments, suggesting a possible drain of nutrients resulting from the presence of the biostimulant rather than a benefit. Furthermore, negative responses in terms of biomass and morphology were associated with some biostimulants in P-amended conditions. Clear differences were observed in fungal community structure after biostimulant treatments were applied, with separation between communities, suggesting initial divergence in the microbial community after inoculation. Some genera associated with plant growth-promotion including *Trichoderma* persisted in the soil after fungal biostimulant treatments were applied, although these treatments were only associated with improved crop growth in high-P scenarios and were not linked to improved crop P uptake. The longer-term implications of these effects require further research and should be corroborated by field experiments.

4.2. Introduction

Biostimulants may contribute to improved crop yield, but can also offer additional benefits such as improved resistance to biotic and abiotic stresses (Frioni *et al*., 2018), and are now being used on a large scale, with a global market value projected to reach $2 billion by 2020 (New Ag International, 2019). An increased interest in the potential of fungi (specifically arbuscular mycorrhizal fungi) to be used to improve phosphorus mobilisation in agricultural contexts has been observed by Rouphael *et al*. (2015). The use of plant growth-promoting microorganisms in the form of biostimulants in agriculture has been advocated as a valuable component of integrated nutrient management regimes (Ruzzi *et al*., 2015; Vassilev, 2015). Fungal biostimulants may offer various opportunities to address nutrient management issues, and there may also be financial advantages to incorporating them into food production methods. It has been reported that biostimulants are most efficacious when used in conjunction with integrated nutrient management regimes (Rouphael *et al*., 2015).

One of the most unclear aspects of biostimulant activity is how to identify modes of action by which plant growth may be enhanced. In addition to increasing levels of plant-available nutrients in the rhizosphere, both organic and inorganic fertiliser amendments affect the community structure and composition of soil microorganisms (Marschner *et al*., 2003). As the structure and composition of rhizosphere microbes change, plant growth may be correspondingly influenced. A variety of factors have been known to be responsible for this, including increased nutrient turnover, induced disease resistance, competing for active sites on root tissue, interfering with pathogen signalling hormones, and disease suppression (Storer *et al*., 2016), which are all very much subject to the seasonal effects and the timing of fertiliser application (Di Salvo *et al*., 2018). Evidence for a variety of fungi-derived plant growth-promoting effects is being increasingly documented, and which may include imprpved root morphology and increased plant nutrition through phosphorus mobilisation (Sanchez-Evesta *et al*., 2016). Rouphael *et al*. (2015) identify the need for research which improves understanding of interaction between fungal species, crops and environment for the purposes of improving the efficacy of proposed biostimulant formulations. The method used in the present study seeks to quantify morphological differences between plants treated with biostimulants and then contrast this with plant-available P in treated soils, and in so doing isolating the stages of the mode of action responsible for plant growth-promotion. The approaches used to characterise the phenotypes of a given species (phenomics) have been developed to overcome restrictions of traditional plant morphology measurement methods (Duan *et al*., 2018), and techniques now include novel image sensors, imaging analysis and robotics. The capability of this infrastructure to facilitate high-throughput and large-scale, non-invasive phenotyping may be used in addition to reduce the time and inaccuracy of traditional

measurement methods, which are often manual. Visible light imaging technology, due to low cost and ease of implementation, is used widely for plant imaging. Through recording similar wavelength perception as in human vision (400-700 nm), 2D photography can detect useful information for the analysis of leaf biomass, yield and leaf morphology. In addition, Rouphael *et al*. (2015) highlighted the importance of identifying molecular mechanisms responsible for plant growth-promoting effects in commercial biostimulants. Owen *et al*. (2015) observe the complexities involved in determining whether the presence of a biostimulant is efficacious in crop growth-promotion, with interactions between microbial communities and crops varying depending on species composition, soil nutrient regime, temperature, pH, seasonality, and organic inputs.

Although a great deal of research has been carried out into plant growth-promoting microorganisms and their possible benefits that could be commercially exploited (Calvo *et al*., 2014; Ruzzi *et al*., 2015; Vassilev, 2015; Rouphael *et al*., 2015), a great deal remains unknown. In particular, evidence concerning the dormancy, lifespan and viability of biostimulants remains vague and largely inconclusive (Kaminsky *et al*., 2019). The biotic and abiotic effects of different sites on the viability of biostimulants also remains largely unexplored (Hart *et al*., 2018), and an integrated method of formulating biostimulants that are tailored to specific sites, while much advocated has yet to be devised. In any case, tailored biostimulants offer additional cost challenges in terms of any approach, which must rely on gauging a number physical, chemical and biological properties of the target soil, and cost-effective means of doing so are yet to be developed (Savy *et al*., 2020; Santini *et al*., 2021).

The large majority of commercial biostimulants that are designed to improve crop growth using fungi claim to do so by introducing AMF to the rhizosphere (Calvo *et al*., 2014). However, Glomermycota are by no means the only fungal phyla capable of offering plant growth-promoting potential, with soil yeasts, Zygomycota and Mucormycota also containing taxa that have demonstrable plant growth-promoting properties (Detheridge *et al*., 2016). Biostimulant formulations often contain microorganisms which are selected based upon their performance in controlled laboratory screenings and their potential for large scale cultivation, rather than their survival or functional potential in field environments (Kaminsky *et al*., 2019), and the methods used in culturing and refinement of biostimulants, particularly in consortia treatments, mean that a considerable number of microorganisms may be being introduced to the rhizosphere from biostimulant treatments (Santini *et al*., 2021). As such, it could be the case that a biostimulant is having a beneficial or negative effect on a crop that is microbially mediated by entirely different mechanisms and by different species to those advertised.

It is also largely unknown what biostimulant effects are on resident microbial and plant communities, specifically in terms of persistence, niche overlap and legacy effects (Parnell *et al*., 2015; Kaminsky *et al*., 2019; Hart *et al*., 2018). There remain large knowledge gaps as to the extent to which biostimulants may introduce genetic material to resident microbial and plant communities, and what processes govern the exchange of genetic material. In terms of persistence and degradability, biostimulant manufacturers often claim explicitly that the mycorrhizal fungi or PGPB used in formulations are naturally present in the environment (Symbio, 2019). If this is the case, it remains unclear whether the supposed effects produced by the biostimulant would not have arisen from natural ingress of microbes from the environment anyway, or what the value actually is in terms of increasing the relative abundance of naturally occurring microbes. In spite of claiming this, the factors that influence the persistence, degradability, proliferation and spread of biostimulants are also largely unknown (Hart *et al*., 2018).

This study uses a number of approaches to address this knowledge gap. The literature has focused to a large extent on AMF species for plant growth promotion (Fester and Sawers, 2011; Smith and Read, 2010; Zuccaro *et al*., 2014), and other fungal phyla have been relatively unexplored in terms of commercial exploitation, and increasing evidence is emerging that the supposed benefits of attempting to manipulate AMF abundance in agricultural environments have been greatly overstated (Ryan and Graham, 2018). It has been suggested above that biostimulants may affect crops by different mechanisms and species to the intended ones, and in the case of the AMF biostimulant used in this study, initial investigation using simple culturing and molecular diagnostic methods (unpub.) revealed the presence of a number of fungal species from other phyla which were likely included in the biostimulant as a result of the culturing process. It is possible that purported growth improvements observed in crops treated with this biostimulant may not be due to mycorrhizal symbiosis, but could be due to other fungal taxa with lesser known effects (Detheridge *et al*., 2016). A means of investigating this would be to inoculate a non-mycorrhizal species with this biostimulant and examine the behaviour of other microbes in relation to plant P uptake, and this approach has been undertaken in this study, with a non-mycorrhizal experimental crop (*Arabidopsis thaliana)* used to examine the extent to which biostimulant efficacy is due to specified properties. The experimental method sought to do this by measuring morphological variables associated with crop performance, and measured P uptake to ascertain to what extent mineral nutrition was influenced by the experimental conditions. The use of phenomics methods to examine the efficacy of biostimulant application was previously explored in Chapter 3 on *Lolium* plants. This method was limited in terms of the data it was able to collect (restricted to plant height, area, width, perimeter and compactness). The choice of *Arabidopsis* as a model

plant in the present study has been selected in order to overcome this, in addition to its physiological relationship to oilseed rape (Santaniello *et al*., 2012). Rosettes are measured as opposed to the entire plant structure, which is intended to reduce the issues discussed in Chapter 3 associated with sideways-positioned cameras in 2D phenomics imaging. Unlike Chapter 3, this study involves autoclaved (sterilised) controls of all three biostimulants. This allows the effects of the microorganisms used in these formulations to be examined for their efficacy. In Chapter 3, only one biostimulant had a sterilised control, which provided evidence to suggest that in some instances, live biostimulants are less efficacious than when sterilised. The aim of the present study is to develop the method further by including sterilised controls of all biostimulants used.

Many factors influence the structure of fungal communities, including nutrient availability, pathogen presence in the rhizosphere, availability of active sites on root tissue, but mainly pH (Tian *et al*., 2017). The evidence of fungi-derived plant growth-promoting effects continues to be documented, and include increased root proliferation and surface area and improved plant nutrition through phosphorus mobilisation (Sanchez-Evesta *et al*., 2016). As well as bacteria, fungi can solubilise and chelate inorganic nutrients, allowing them to be available for plant uptake (Zhang *et al*., 2014). Detheridge *et al*. (2016) discuss the need to understand the complexities of the interactions between plants, the soil and microbes, their roles in plant nutrition, soil function and how they may be affected by soil nutrient status, temperature, pH and organic inputs as a result of agricultural management practices. Organic and inorganic fertiliser amendments are well-understood to affect the community structure of soil microorganisms (Marschner *et al*., 2003), which has resulting implications on crop growth. Rouphael *et al*. (2015) identify the need to research the interaction between fungi, crops and abiotic variables. Similarly, Dias *et al.* (2015) highlight that a better understanding of the role of microorganisms in plant nutrition and nutrient cycles in agricultural contexts is needed, and stress that the interactions between fungi, plants and other soil microbial communities are of particular importance. Generating useful results at the appropriate large data scales is becoming increasingly possible through high-throughput metagenomics (Carbonetto *et al*., 2014), and in so doing, allowing us to better isolate the modes of action responsible for plant growth-promotion. Rouphael *et al*. (2015) also identify the importance of elucidating the molecular mechanisms involved in plant growth-promoting effects in commercial biostimulants. The approach used in this study involved examining fungal DNA in the rhizosphere of treated plants, which allows us to suggest which fungal species persist in the soil after biostimulants have been applied, and the nature of fungal community structure after inoculation. This information is essential for the purposes of determining the persistence and

longevity of the molecular mechanisms involved in plant growth promotion following biostiumlant application (Rouphael *et al*., 2015).

4.3. Materials and methods

4.3.1. Experiment design

Seeds of *Arabidopsis thaliana* (ecotype Col-0) were germinated on a wet filter paper, and then sown in 6 cm diameter pots (vol 283 cm^3, n = 140) with W. island 13 compost, a low lime-buffered pH peat. *Arabidopsis* was selected as the experimental model crop on account the detailed modelling of its growth pattern presented in scientific literature, which provides the clearest understanding of when morphology is altered as a result of a treatment (Pavicic *et al*., 2017). The growth media consisted of 2:1 compost: sterilised sharp sand (details presented in Chapter 3). To prepare the nutrient conditions, soil added to half of the pots was blended with 0.092 g ground phosphorus fertiliser (47 % P), in accordance with RB209 recommendations for brassica production on low P soils (P index 0, between 0–9 mg l^{-1}), meaning each container received 0.043 g of plant-available P. The remaining 70 containers received no P amendments. The *Arabidopsis thaliana* seeds were not surface sterilised.

One seedling was placed in each growth pot, and biostimulant treatments were applied in powder form to the entire surface area of the potting substrate one week after sowing, and watered into the soil using approx. 100 ml of water per pot. The experiment design involved ten replicates of each biostimulant, which were applied one week after seeds were planted. The biostimulants were Biagro Brassika (KGB), Umostart Rhizobium (UR), Umostart Mycorrhizae (UM) applied at approx. ×400 the recommended application rate, which was to account of the fact that the small volumes of the recommended application rates which would not have been practical for the surface area of growing pots. Sterilised controls of each biostimulant were also used in the trial (elemental composition summarised in Table 4.1). The method of sterilising the biostimulants consisted of autoclaving them (121 °C, 100 kPa, 90 minutes) to help ascertain nutrient effects when contrasted with the live treatments. Controls consisted of P fertiliser with no biostimulant, and ten replicates of a control with no added P (Control-P), giving n=10 for 14 total treatments. The 140 growth containers were placed into a tray on a conveyor system, with the positions of the pots on the conveyor system trays completely randomized, with positions assigned using a random number generator. Pots were weighed daily and watered to 75% field capacity. Growth conditions consisted of 20°C daily temperature and 18°C nightly temperature. Day-lengths were maintained at 14 h using 600 W sodium/LED lamps (intensity = 260 µmol m^{-2} s^{-1} PAR). Plant hygiene was maintained by visual inspection throughout the experiment. The experiment duration was 40 days. Further details

concerning the method of automated weighing, watering and data collection are presented in Chapter 3, along with a description of the intended uses of the bioinoculants.

Table 4.1a: Descriptions of treatments, composition and function of commercial biostimulants used in study. All biostimulants were stored and applied in powder form.

Biostimulant	Acronym	Carrier	Biology
Biagro Brassika + P	KGB+P	Soluble powder	*B. amyloliquefaciens, B. subtilus, B. lichenoformis, B. circulans, B. megatererium, B. pumilus, B. coagulans*
Biagro Brassika - P	KB-P		
Sterilised Biagro Brassika + P	KB(S)+P		*Sterilised*
Sterilised Biagro Brassika - P	KB(S)-P		
Humostart rhizobium + P	UR+P	Micro-ground DAP plus zinc, suspended in mono-potassium phosphate and di-potassium phosphate with zeolite	Several phosphate-mobilising & N fixing genera of bacteria (listed as *Bacillus, Paenibacillus, Azotobacter, Azospirillum* and *Beijerinckia*)
Humostart rhizobium – P	UR-P		
Sterilised Humostart rhizobium + P	UR(S)+P		*Sterilised*
Sterilised Humostart rhizobium – P	UR(S)-P		
Humostart mycorrhi-zae + P	UM+P	Micro-ground DAP plus zinc with zeolite and AMF blended through	Unconfirmed but very probably contains *Trichoderma, Glomus interadices, G. mosseae, G. a-ggregatum* and *G. etunicatum*
Humostart mycorrhi-zae - P	UM-P		
Sterilised Humostart mycorrhi-zae + P	UM(S)+P		*Sterilised*
Sterilised Humostart mycorrhi-zae - P	UM(S)-P		

Controls used in this study	
Control + P	Potting substrate with RB209 recommended P amendment
Control - P	Potting substrate with no P amendment

Table 4.1b: Elemental composition (%) of selected commercial biostimulants (Medac Ltd, 2019). Various reasons prevent disclosure of the full composition all biostimulants used in this study

Biostimulant	C	H	N	NO_2^-	NO_3^-	PO_4^{3-}	SO_4^{2-}
KGB	35.19	5.71	2.15	<0.10	<0.10	2.58	0.15
UR(S)	0.22	4.55	10.29	<0.10	<0.10	77.42	5.08
UM(S)	0.24	4.46	10.29	<0.10	0.17	71.23	4.37
UR	0.19	4.65	10.40	<0.10	<0.10	72.45	5.28
UM	0.17	4.31	10.59	<0.10	0.25	75.49	4.69

Table 4.1c: Biostimulant recommended application rates and experimental application rates. Quantities applied were between 1 and 400 times the recommended application rate per hectare to account of the fact that the small volumes of the recommended application rates which would not have been practical for the surface area of growing pots. Autoclaved controls were applied at the same rates as live treatments.

Biostimulant	Recommended application rate (kg ha^{-1})	Experimental application rate (g)
KGB	0.15	0.15
UR	0.15	0.15
UM	0.15	0.15

Characterisation of the substrate used in the trial is described in Table 3.2a and 3.2b of Chapter 3. To avoid confusion, all mentions of “soil” in this report refer to the substrate described here.

4.3.2. Data collection and analysis

The plant phenotyping facility at the National Plant Phenomics Centre (Plas Gogerddan, Aberystwyth University, Wales) was used for plant morphotyping of *Arabidopsis* plants undergoing biostimulant treatment. Using a similar method to the approach described in detail

in Chapter 3, test specimens were imaged daily by overhead CCD camera for RGB images, positioned in a PlantScreen™ analysis chamber with plants transported on an automated conveyor system between imaging, weighing and watering stations. RGB images were obtained, from which segmentation and total projected leaf area were calculated by transforming original RGB images to HSI colour space. Raw parameters of rosette area and perimeter were obtained by using fixed thresholds to identify background pixels and plant pixels, creating binary images of the plant rosettes. Regions with areas registering less than a predefined threshold were removed. The binary images were used for calculating parameters of area and perimeter. The total rosette area was calculated as the total number of pixels and then transformed into millimetres. Using IMAQ Edge Detection VI, the rosette edge was extracted, and the perimeter values could therefore be obtained by calculating the number of pixels forming the rosette edge, and then transformed into millimetres. This method is based on the approach described in Fischer *et al*. (2016).

This process enabled the area of experimental crop rosettes to be calculated, as well as the perimeter. Area was selected as the most informative overall measure of growth that could be inferred from morphological measurements, as it could be directly linked to biomass (whereas perimeter, circularity, symmetry and eccentricity parameters cannot). The smallest convex hull which contained each rosette was detected using OpenCV, which calculated pixel counts for each plant. From these values, the ratio of rosette area to convex hull area was used to calculate compactness scores. The parameters of rosette perimeter, area and extracted convex hulls were used for calculations of further rosette morphometric parameters (roundness1, roundness2, isotropy, eccentricity, rotational mass symmetry (RMS), and slenderness of leaves (SOL)) (PlantScreen analyzer™, PSI, Czech R.).

To characterise detailed morphology of treated plants, morphological parameters were grouped into four general categories based on the general characteristics they measure, following the approach described in Pavicic *et al*. (2017). These categories were growth measurements, circularity, symmetry and centre distance. These parameters were then compared over time. Growth measurements consisted of area and perimeter (method described in Chapter 3). Roundness1 and roundness2 and isotropy constituted the circular parameters. Roundness1 refers to the comparison between measured rosette area and a perfect circle with the same perimeter. These variables are controlled by slenderness and perimeter of the leaves and petiole length. In wild plants, roundness1 usually measures 0.1 and 0.5 (Pavicic *et al*., 2017), and a perfect circle has a value of 1. Roundness2 compares the area of rosette convex hulls with plant perimeter, and wild plants are reported to measure between 0.7 and 1.0 for this ratio. Isotropy measurements involve imposing a polygon over the rosette, and therefore behaves in a similar manner to roundness 2 but with a decrease in

tendency. Circularity parameters, while not directly linked to biomass, can nevertheless provide information on the onset of the transition from vegetative to reproductive growth, the determining of which can have major effects on biomass accumulation (Camargo *et al*., 2016).

The symmetry parameters were eccentricity and RMS. Eccentricity is a measure of the elliptical tendency of a plant rosette, with higher values representing sharp rosettes, and lower values representing rounder rosettes. In wild plants, a peak in eccentricity is observed which will decay with time, with possible smaller peaks later in the growing period. This describes an oscillatory pattern between a round and a sharp rosette. RMS is calculated via a ratio between the non-overlapping area of the rosette convex hull and a perfect circle sharing the same area and centre point, and the overlapping area of both shapes. This variable typically follows similar trajectories to that of eccentricity, but peaks may be sharper and relative values may be higher.

The centre distance parameters were compactness and SOL. Compactness calculated via the ratio between rosette area and rosette convex hull area. Compactness ratios describe petiole length in relation to the width of the leaves. SOL is calculated via the ratio between squared rosette skeleton and rosette area. It describes leaf blade sharpness, and depends on the number of leaves. In wild plants, this ratio typically ranges between 0 – 50 (Pavicic *et al*., 2017).

4.4.3. Phosphorous measurements

At the end of the experiment (40 days post-inoculation), the above ground biomass of the plants were harvested (consisting of the rosette and shoot), and were subsequently dried for 2 days at 70 °C to determine values for dry biomass, along with manual measurements of plant height. The roots were washed from the potting substrate under cold tap water. Fifty grams of representative (thoroughly homogenised) potting substrate from each pot was dried at 70 °C for 3 days. Dried leaf and potting substrate samples were milled to fine powder using a Foss CT193 (Cyclotec) before further elemental analysis. P concentration in plant leaf tissues was obtained from 1 g of a milled sample of plant shoots. After milling, the samples were ashed in a 500 °C furnace for 12 hours. The ashes were then dissolved in 1 ml of concentrated (14 M) HCl and made up to 50 ml with deionised water. Plant tissue orthophosphate concentrations were then measured using the Olsen P method (Murphy & Riley, 1962). Soil from the experimental pots was extracted using acetic acid and tested for plant-available P using a colorimetry method (Murphy & Riley, 1962).

4.3.4. Substrate preparation and DNA extraction

A 200 g sample of compost potting substrate from each of the 14 treatments used in the *Arabidopsis* pot experiment were frozen at -80 °C the same day as plants were removed from the conveyor system (Day 40). Substrate was removed from the roots by gentle shaking followed by carefully removing volumes of substrate that had aggregated to the roots manually using sterile, nitrile gloves. Frozen substrate samples were freeze-dried for 48 hours and ground (< 1 mm). Sieved substrate was thoroughly mixed to ensure homogeneity and to reduce the possible confounding effects of microscale community variability (Penton *et al*., 2014), and 200 mg was added to DNA extraction tubes. DNA was extracted using a PowerSoil™ DNA extraction kit (MoBio Laboratories, Solana, CA, USA), following the manufacturer's instructions. The D1 region was selected as the target region, as it provides good resolution to genus level for the majority of fungal taxa (Cole *et al*. 2014). The D1 variable region (approximately 200 base pairs) of the large sub unit (LSU) of ribosomal DNA (rDNA) was amplified using fungi specific novel primers; D1F2 (CYYAGTARCTGCGAGTGAAG) and the reverse NLC2AF (GAGCTG-CATTCCCAAACAA). The forward primer was linked at the 5' end to a barcode sequence, and a separate barcode was used for each sample to allow for multiplexing during sequencing. The forward primer was calibration sequence and an Ion Torrent™ adaptor, and the reverse primer was linked at the 5' end to an Ion Torrent™ adaptor.

Amplification was performed in a 50 µl Polymerase Chain Reaction (PCR) using Promega GoTaq G2, DNA polymerase (Promega, Madison USA). Each reaction contained 250 nM of each primer, 1 mg ml-1 BSA, 200 µ M dNTPs and 0.5 U of DNA polymerase in the buffer. The primer D1F2 was linked at the 5' end to the IonTorrent adapter sequence (CCATCTCATCCCTGCGTGTCTCCGAC), a TCAG key and an IonXpress Barcode. Primer NLC2AF was linked at the 5' end to an Ion Torrent adapter sequence (CCTCTCTATGGGCAGTCGGTGAT). The PCR conditions were 94 °C for 5 min (initial denaturation) followed by 30 cycles at 94 °C, 30 s (denaturation); 52 °C, 30 s (annealing); 72 °C, 30 s (extension) and a final extension step at 72 °C for 5 min. The PCR reactions were cleaned using spin columns (NBS Biological, Huntingdon UK) and the amplified DNA was quantified using NanoDrop (NanoDrop Products, Wilmington USA). Emulsion PCR was carried out using Ion-Torrent 200 bp template kit using the Ion Torrent One Touch 2 system following the manufacturer's instructions. Amplified sequence particles were enriched using One Touch ES to remove non-template particles, and were then sequenced on "316" (100 Mbp) microchips using the Ion Torrent Personal Genome Machine (Life Technologies, Waltham USA).

4.3.5. Sequence data processing

DNA sequencing data were downloaded from the Ion Torrent™ server in BAM format (a binary version of sequence alignment map). Data were unpacked from BAM to FASTA (standard text format) and QUAL (quality information) files, using the PICARD software package. Sequence data were quality checked and trimmed to 200 bp. Demuliplexing was carried out using MOTHUR (v. 1.31.2; (Schloss *et al*., 2009)). Sequences with mismatching barcode and primer sequences (fewer than 100 bp) were discarded. Sequences were checked for putative chimeric sequences through the UCHIME algorithm (Edgar *et al*., 2011) against a reference database of curated fungal LSU sequences obtained from the Ribosomal Database Project (RDP) website (Cole *et al*., 2014). OTUs (operational taxonomic units) were assigned using USEARCH / UPARSE (v7 (Edgar 2013)) at 97 % clustering (Detheridge *et al*., 2016). Clusters containing fewer than 10 sequences were discarded. A taxonomy was assigned to each OTU using the Naïve Bayesian Classifier against a curated fungal database (Wang *et al*., 2007).

Where genus could not be assigned using the classifier, an OTU identifier was assigned to that cluster. Data were then rendered in Excel and standardised by dividing the number of reads of each OTU by the total number of fungal reads in each sample. This provided relative abundances of assigned taxa for each sample. Non-fungal taxa were reported separately. Shannon diversity index, where P_i = relative proportion of the *i*th taxa) and Simpson diversity index, were calculated for each sample (Equations 1 and 2).

Equation 1: Shannon diversity index

$$\left(-\sum_{i=1}^{s} P_i ln P_i\right)$$

Equation 2: Simpson diversity index

$$\left(1/\sum_{i=1}^{s} P_i^2\right)$$

The broad ecological function of the fungi identified was assigned to each taxon at genus or family. If different ecological functions could be identified within a taxon, a function was only assigned when more than 75 % of known species within the taxon could be assigned to a single function. Otherwise, the function remained undetermined (Detheridge *et al*., 2016). Five ecological groupings, primarily associated with plant roots were identified: SAP (saprotrophic fungi), PATH (pathogens), AMF (arbuscular mycorrhizas), DSE (dark septate endophytes) and CHEG (four taxa associated with grassland habitats, Griffith *et al*. (2002)).

4.3.6. Statistical analysis

The data were summarised using R Studio, and visualised using dplyr and ggplot2 packages. Local regression curves were plotted for the measure of each variable each day, and the ggfortify package was used to determine which statistical model would be most suitable to analyse each variable. To test the effect of replications, time period and biostimulant treatment, a statistical approach involved two-sample Kolmogorov-Smirnov tests between the Control+P treatment and the other biostimulant treatments across the nine interpretive variables described above, applied at 95 % confidence level. PSI generated image analysis data was carried out for the 40 day period, and results are presented in the form of local regression curves displaying data for each morphometric variable during the experimental period. Shaded areas show extent of standard error around the mean, calculated using the method.args function of geom_smooth in R. In addition to this, manual measurements were taken for plant height, wet mass and dry mass after the plants were removed from the conveyor system. A Multivariate Analysis of Variance (MANOVA) was carried out to ascertain the effects of P amendments and sterilising biostimulant treatments on the measured growth variables. Prior to analysis, the Shapiro-Wilk test was used for multivariate normality. The relative abundance of fungal genera were also visualised using the R Studio package using non-metric multidimensional scaling (NMDS) ordination to identify patterns in the data based on the Bray-Curtis similarity index. Analyses of variance (ANOVA) tests were performed on OTU abundances between treatments. Phylum percentage data were log transformed and data checked for normality (Shapiro-Wilk). All results were considered significant at the $p < 0.05$ level.

4.5. Results

Multivariate Analysis of Variance (MANOVA) analysis demonstrated that both P amendments and sterilising biostimulants had highly significant effects (MANOVA, $p < 0.0001$). Two-sample Kolmogorov-Smirnov tests between the Control+P treatment and the other biostimulant treatments revealed significant differences between treatments across all variables (Table 4.2).

Table 4.2: Summary of P values between Two-sample Kolmogorov-Smirnov tests carried out between all biostimulant treatments and the Control+P treatment across the nine variables measured. Asterisks denote levels of significance. P-values below 0.05 are flagged with one asterisk (*), p-values below 0.01 are flagged with two asterisks (**), p-values below 0.001 are flagged with three asterisks (***)

	Area	Perimeter	Roundness1	Roundness2	Isotropy	Compactness	Eccentricity	RMS	SOL
KB+P	***0.01****	0.1	0.2	***0.03****	***0.03****	***0.05****	***0.004****	***0.02****	0.17
KB-P	0.17	0.23	***0.04****	0.17	0.23	0.77	0.11	***0.02****	0.06
KB(S)+P	***0.0002****	***0.004****	0.77	0.12	0.16	***0.01****	***0.001****	***0.02****	0.08
KB(S)-P	0.43	0.3	***0.03***	0.51	0.7	0.13	***0.02****	***0.007****	***0.01****
UR+P	***0.002****	***6.54E-05****	***0.03****	***1.62E-05****	***3.22E-10***	***1.61E-05****	***7.45E-12****	***4.67E-06****	***0.004****
UR-P	***8.94E-12****	***2.87E-06****	0.16	***0.0008****	***4.04E-05****	***0.02****	***7.23E-05****	***0.0008****	***1.95E-09****
UR(S)+P	***0.02****	***0.005****	***0.04****	0.69	***1.46E-06****	***0.05****	***0.03****	0.24	***5.1E-07****
UR(S)-P	***2.2E-16****	***2.2E-16****	0.06	***2.2E-16****	0.15	***2.2E-16****	***2.2E-16****	***2.2E-16****	***2.2E-16****
UM+P	***1.03E-05****	***0.003****	0.45	***0.0004****	***0.002****	0.19	0.16	***0.02****	***3.21E-07****
UM-P	***1.33E-07****	***1.15E-08****	0.73	***3.44E-05****	***3.25E-06****	***2.41E-06****	***5.26E-06****	***0.0003****	***5.81E-09****
UM(S)+P	0.37	***2.14E-06****	***0.0004****	0.97	***2.69E-07****	***0.02****	***0.02****	0.36	***2.59E-12****
UM(S)-P	***2.2E-16****	***2.2E-16****	0.12	***1.55E-15****	***0.003****	***1.24E-08****	***5.86E-13****	***1.95E-14****	***1.44E-15****
Control-P	***2.72E-07****	***9.43E-13****	***0.003***	***0.02****	0.4	0.07	***0.005****	***0.03****	***2.27E-05****

4.4.1. Growth measurements

To analyse the effects of the biostimulant treatments and phosphorus amendments, local regression curves were plotted. This analysis confirmed earlier observations of significant differences in growth and development between treated plants. Sterilised UR with added P showed significantly slower growth, reduced rosette area and perimeter compared to the controls and other biostimulants across the experimental period (Figures 4.1a and 4.1b), but live UR treated plants developed significantly faster than other plants in P amended soils. The UR and UM treatments share the same carrier, but have different biofixed plant growth-promoting microbes, and in the case of P-amended treatments, the rhizobia appeared to offer greater benefits to the plant than the AMF treatment, which is not unexpected as the experimental crop was non-mycorrhizal. In P-amended treatments, the live UM treatment produced significantly delayed growth and less overall growth than the control. Significantly greater overall growth was achieved by plants which received no additional P treatment (Figures 4.2a and 4.2b). Both UR and UM treated plants grew significantly more vigorously than the controls and KB treated plants in non-P treated soils, although sterilised UM and UR significantly outperformed the live biostimulants. Very similar results were obtained for perimeter measurements although it was less apparent from this parameter the extent to which live UR treated plants outperformed other treatments in P-amended soils.

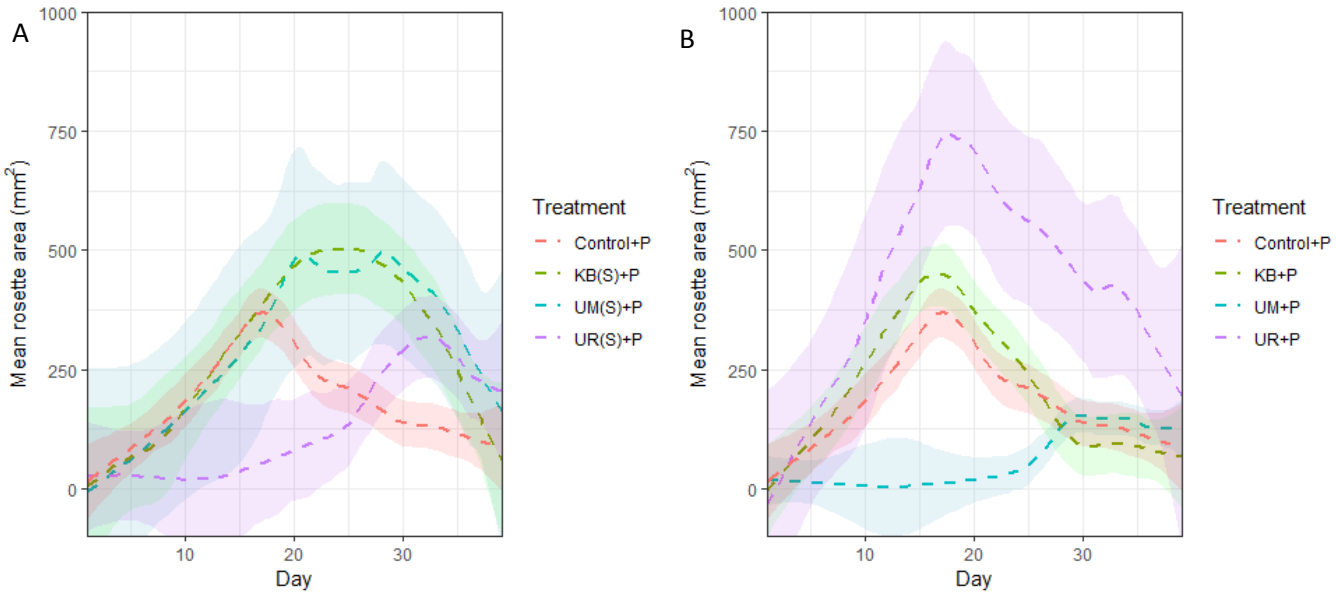

Figure 4.1: Growth measurements for rosette area from day 1 to 40 of P-amended treatments for **(A)** Sterilised biostimulants and **(B)** live biostimulants. Curves denote daily mean; shaded areas denote +/- SE, 95 % confidence interval. n = 10 plants

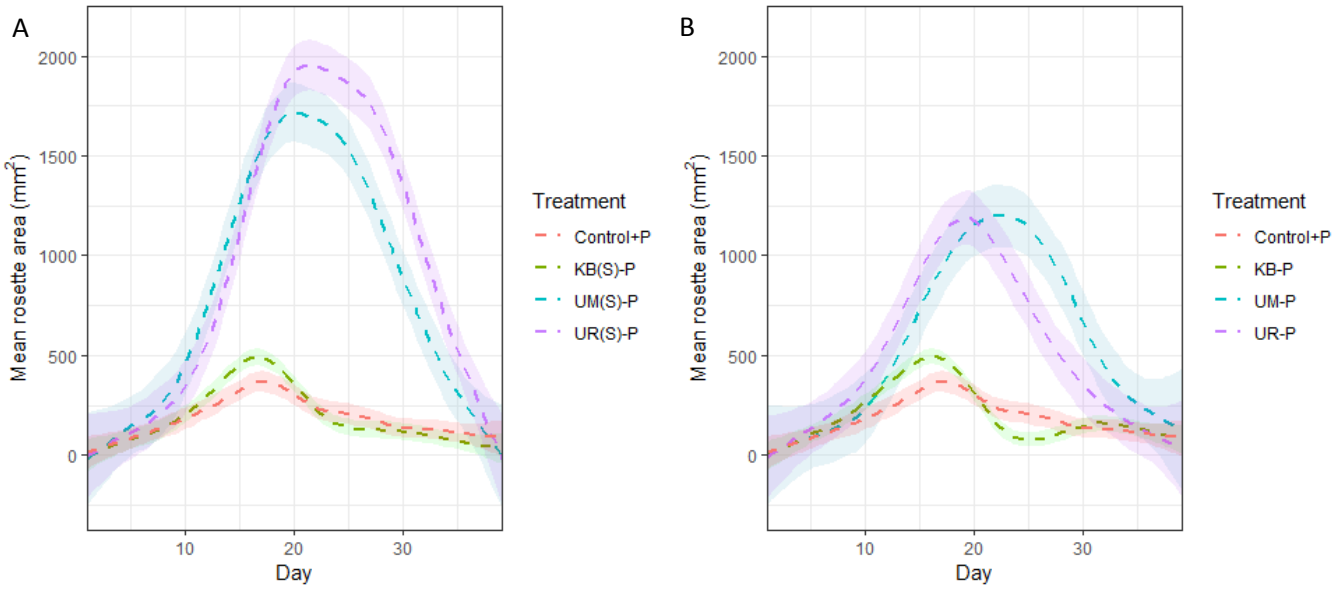

Figure 4.2: Growth measurements for rosette area from day 1 to 40 for non-P amended treatments for **(A)** Sterilised biostimulants and **(B)** live biostimulants. Curves denote daily mean; shaded areas denote +/- SE, 95 % confidence interval. n = 10 plants

4.4.2. Morphological measurements

Morphological data for the circularity parameters of roundness, roundness 2 and isotropy were also analysed (Figure 4.3). Live UM treated plants in P-amended soils showed the highest overall roundness over the total experimental period and in comparison to the controls and the other two biostimulants, and also had the greatest uncertainty. Live UR treated plants in P-amended soils showed the least decrease in roundness over the experimental period, while both live and sterilised KB treatments in non-P treated soils showed the steepest decrease in roundness. Live UR treated plants in P-amended soils also had significantly lower isotropy than the controls and other treatments for the first three weeks of the experimental period. In non-P amended soils, all three biostimulant treatments measured faster declines in isotropy than the controls in both P-amended and non-P amended soils. The morphological parameters that describe symmetry are eccentricity and rotational mass symmetry. For eccentricity, KB treated plants showed the greatest increases in P-treated and non-P treated plants whether sterilised or not. Live UM treated plants showed considerable variability in eccentricity for the first three weeks of the experimental period in P-amended soils. Sterilised UR and UM treatments resulted in less eccentricity than the controls in non-P amended soils, but live treatments produced similar curves to the control. For rotational mass symmetry, sterilised UR treatments in P-amended soils did not follow the usual pattern of a dip followed by a peak, but instead remained relatively constant throughout the growing period (with higher uncertainty than other treatments). Non-sterilised UM followed a similarly aberrant pattern in P-amended soils, remaining relatively constant until the end of the third week of the experimental period. In non-P amended soils, the dip in the curves for sterilised UR and UM was significantly greater than the controls and KB, but live biostimulants produced similar curves to the control. The final morphological parameters analysed were compactness and slenderness of leaves (SOL), which relate to centre distance. In P-amended soils, sterilised and live biostimulants showed a decay in compactness closely resembling the controls, but in non-P amended soils, both live and sterilised UR and UM treatments took longer for compactness to decay than in the controls or KB. Similarly to results seen in the other variables, sterilised UM in P-amended soils had a significantly greater peak in the curve for slenderness of leaves than the control. The other two treatments also exhibited a peak in SOL during the fourth week of the experimental period. Live UR exhibited significantly higher SOL than the other treatments in P-amended soils. In non-P amended soils, UR and UM treatments (both live and sterilised) resulted in significantly higher peaks for SOL than KB and the controls, although sterilised treatments produced significantly higher peaks than live biostimulants.

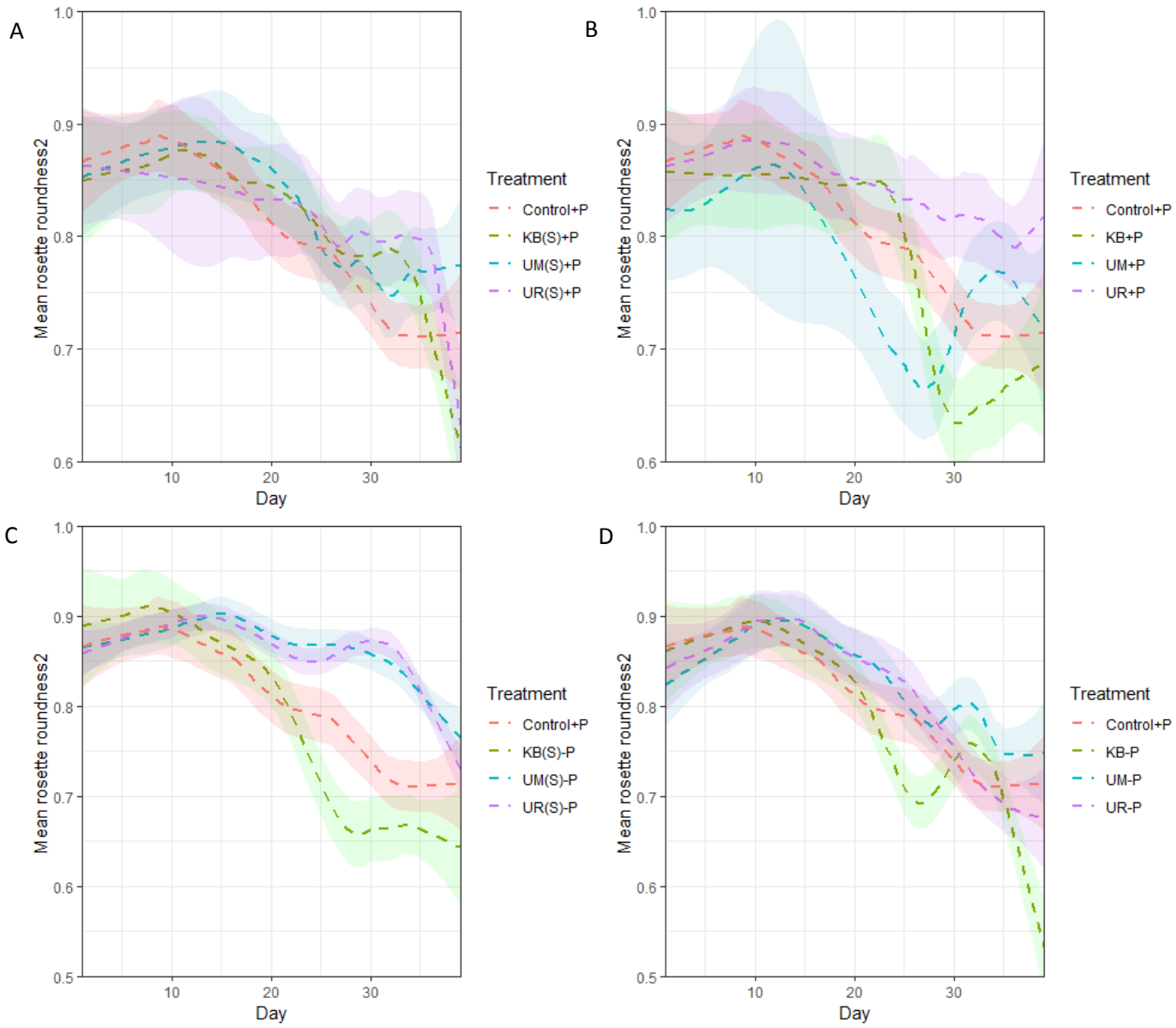

Figure 4.3: Circularity measurements for rosette roundness2 from day 1 to 40. **(A)** Sterilised biostimulants with P amendments, **(B)** live biostimulants with P amendments, **(C)** Sterilised biostimulants without P amendments, and **(D)** live biostimulants without P amendments. Curves denote daily mean; shaded areas denote +/- SE, 95 % confidence interval. n = 10 plants

4.4.5. Phosphorus measurements

There was significantly less plant-available P observed in soil treated with three biostimulants (Figure 4.4, KB-P, KB(S)-P, Control-P). Boxes show extent of upper/lower quartiles around the mean, and whiskers show upper and lower extent of data. Asterisks denote levels of significant variation to the Control+P reference group. P-values below 0.05 are flagged with one asterisk (*), p-values below 0.01 are flagged with two asterisks (**), p-values below 0.001 are flagged with three asterisks (***), and p-values below 0.0001 are flagged with four asterisks (****). Grey circles denote extreme outliers.

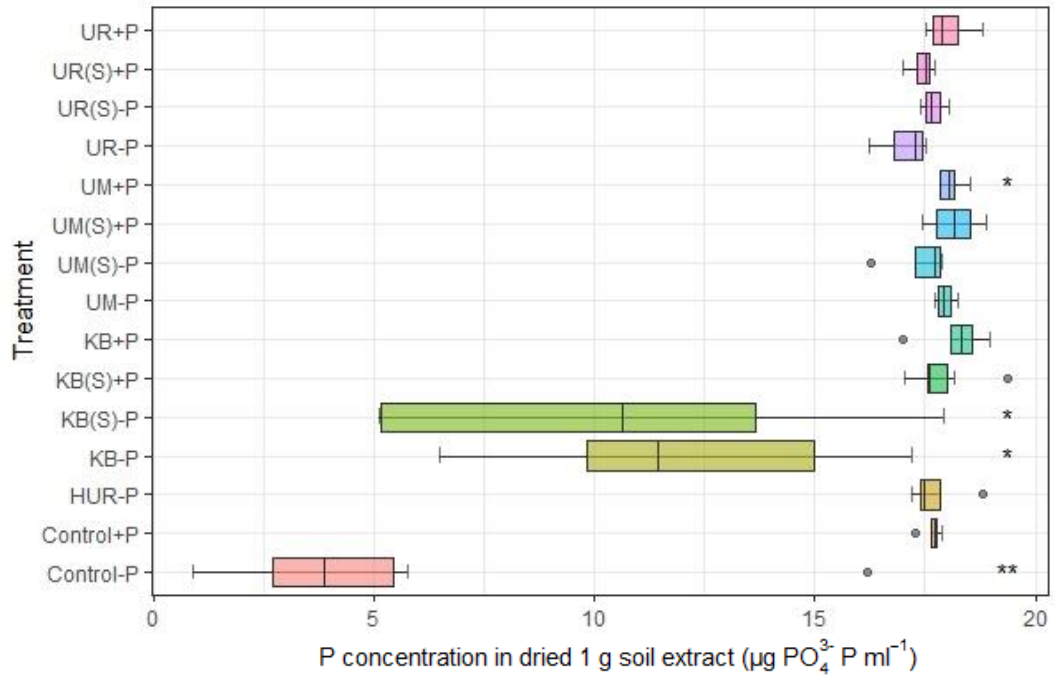

Figure 4.4: Summary of mean PO_4^{3-} (plant-available phosphorus) concentrations obtained from GEN5™ microplate analysis of ten replicates of each biostimulant treatment in 1 g dried soil extracts

After removing results from these three treatments showing significantly lower plant-available P than the other treatments, differences between the remaining treatments became easier to visualise (Figure 4.5). Soil P concentrations of these remaining treatments were fairly similar, with the exception of the UM+P treatment, which was significantly higher than the Control+P reference group. To some extent, these results are fairly close to what was expected, in that the Control-P treatments did not receive RB209 levels of P and was therefore severely P limited, and neither did the live and sterilised KB-P treatments which had less than 3% PO_4^{3-} levels in the carrier substrate. By comparison, the P levels of the live and sterilised UM and UR treatments were expected to be higher as their carrier substrates contained over 70% PO_4^{3-} by elemental composition. However, there was very little difference between the plant available P levels of the soils treated with KGB (live and sterilised) biostimulants and the UR

and UM treatments whether the latter were P amended or not. Therefore, since the latter treatments introduced substantially more P into the soils than KGB treatments through the carrier substrate (~50%), the surplus P must either have been leached from the soil or taken up by the plants.

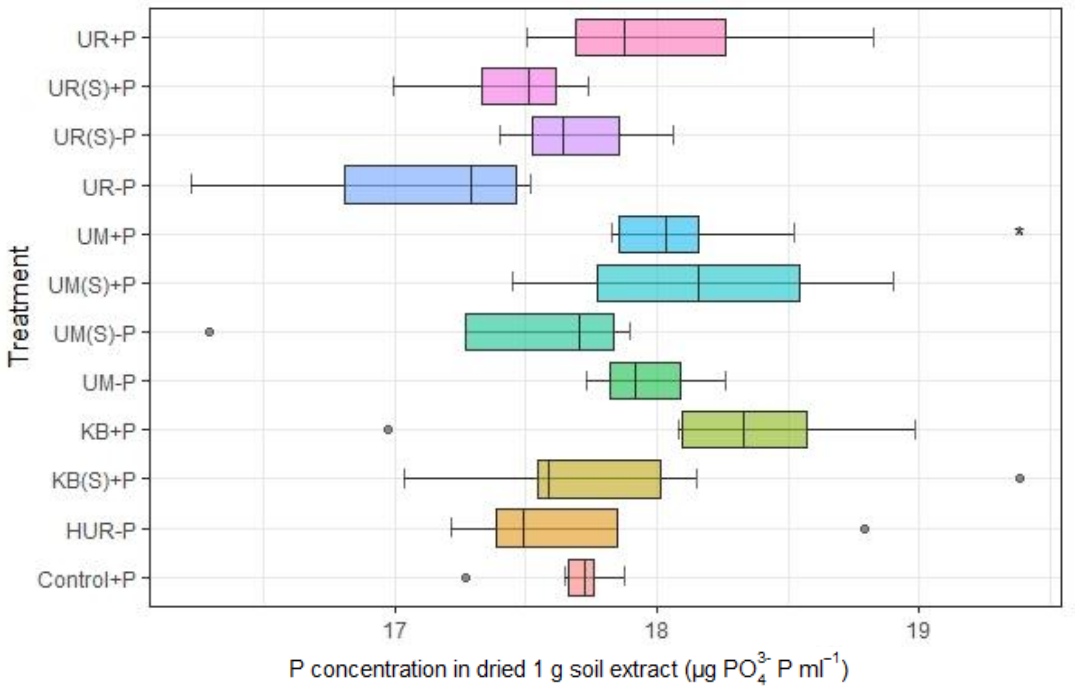

Figure 4.5: Summary of mean PO_4^{3-} (plant-available phosphorus) concentrations obtained from GEN5™ microplate analysis of the replicates of each biostimulant treatment in 1 g dried soil extracts minus the three treatments showing significantly less than the others. Some significant differences between these remaining results are now easier to visualise as opposed to before removing these three treatments

Unlike the findings presented in Chapter 3, lower levels of P was measured in the leaf tissue of plants receiving biostimulant treatments than in the P-amended control, which actually had the highest mean leaf tissue P observed across all treatments (Figure 4.6). This could be evidence of some metabolic cost to the plants from the biostimulant treatments, although it is unclear whether this would be a result of a direct or an indirect processes (such as more carbon exuded to the soil that impacted on the synthesis of ATP, for example). In P-limited soils, plants treated with live biostimulants had higher mean leaf tissue P than sterilised biostimulants, although this was not significant. In P-amended soils, it was the sterilised biostimulants that were more associated with elevated plant tissue P than the live treatments, and was significantly higher in the KGB treatment. In P-limited soils, both the live and the sterilised KGB treatments were associated with significantly lower leaf tissue P than the P amended control, which was not suprising considering how much less plant available P was present in the soils of these treatments (Figure 4.4). The levels of leaf tissue P in plants treated with the live UR-P biostimulant were not significantly lower than the control, however neither

were the leaf tissue P levels of the plants treated with the sterilised KB+P treatment. Overall, no evidence from these findings would be indicative of microbially enhanced crop P uptake.

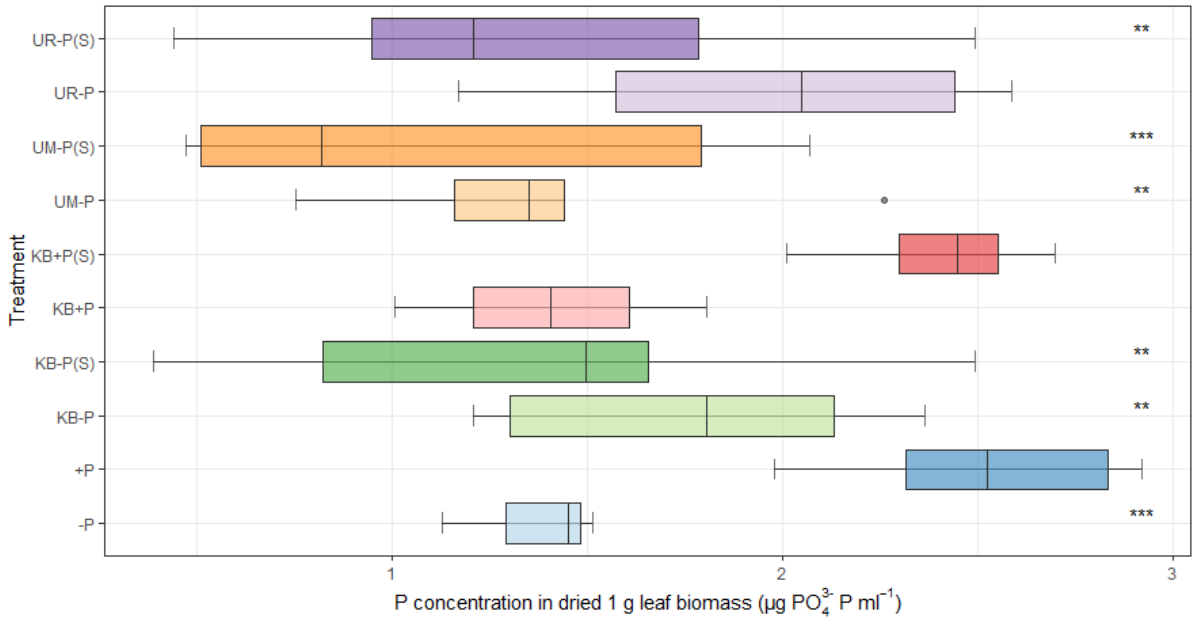

Figure 4.6: Summary of mean PO_4^{3-} concentrations obtained from GEN5™ microplate analysis of the replicates of each biostimulant treatment in 1 g dried leaf extracts. Data could not be obtained from all treatments because plants that underwent some treatments did not have harvestable biomass by the end of the experimental cycle.

4.4.6. Fungal community structure

Across the 14 substrate samples which were analysed, the mean number of sequences obtained after quality control was 28,172 per sample, the lowest being 15,598 (Table 4.3). Of these, >86 % were fungal, the remainder being mostly belonging the genera *Cercomonas* and *Chlorophyta* incertae sedis (of domains Rhizaria and Viridiplantae), which accounted for 23 % and 19 % respectively of the non-fungal sequences cross all samples. General statistics from sequencing run are presented in Table 4.4.

Table 4.3: Summary statistics for sequencing run. All diversity indexes are derived from the equations for Shannon and Simpson indexes, which are given in Equations 1 and 2.

Fungi identified to family %	85
Fungi identified to genus %	66
Ascomycota %	56
Basidiomycota %	7
Fungi incertae sedis %	34
Fungi Total	340579
Non Fungi Total	36831
Inverse Simpson Index (Taxa)	7.57
Shannon Index (Taxa)	2.54
Taxa Count	1666
Inverse Simpson Index (OTU)	8.88
OTU Count	2114
Shannon Index (OTU)	2.84

Ascomycota were the most abundant fungi (mean 56 %; range 17–84 %), followed by Basidiomycota (mean 7 %; range 1–22 %). More than 85 % of fungal sequences were identified to family and more than 65 % identified to genus. The most abundant taxa were the saprophytes *Mortierella parvispora* (mean 22 %; range 3–77 %), *Pseudogymnoascus* (mean 11 %; range 1–26 %), and the ectomycorrhizal *Sphaerosporella* (mean 11 %; range 0–51 %). The most abundant taxa across all samples are displayed in and Figure 4.7, with Figure 4.8 displaying Ascomycota and Basidiomycota abundance.

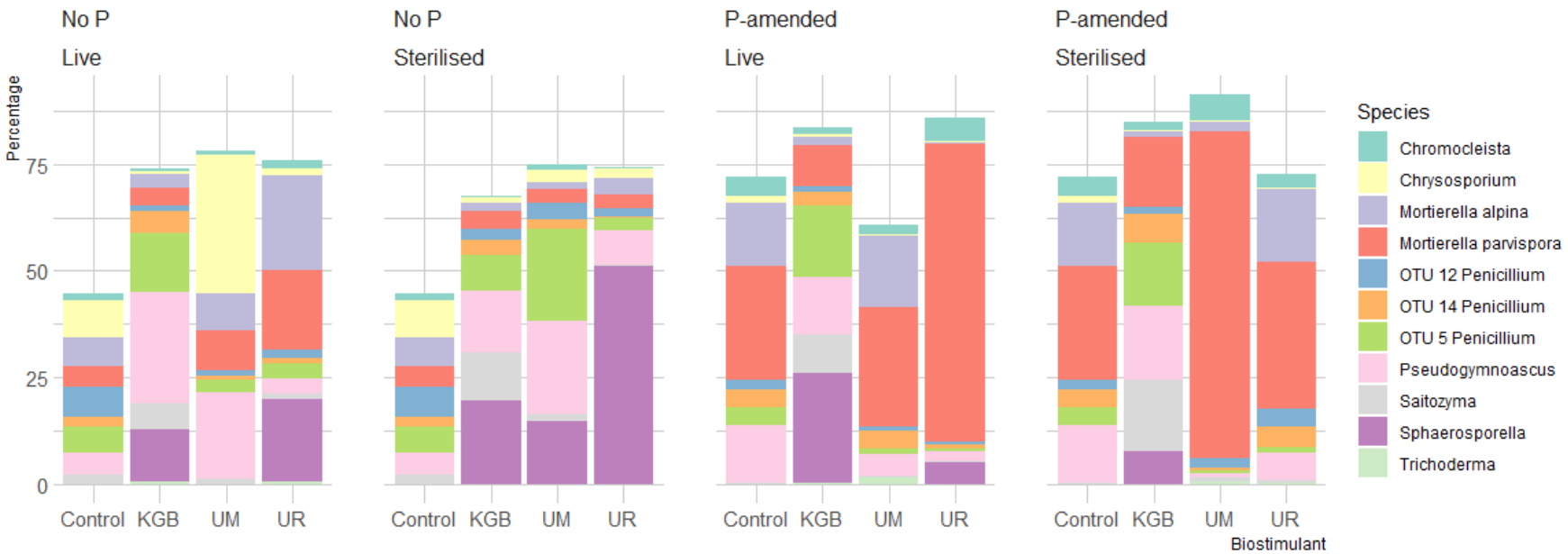

Figure 4.7: Stacked barchart showing mean abundance of the top ten most commonly occurring taxa across the three live and sterilised biostimulant treatments and controls in P-amended and non-P amended substrate (KGB: BiagroBrassiKa, UM: Umostart Mycorrhizae, UR: Umostart Rhizobium)

Table 4.4: Top 20 fungal sequences across all treatments and the mean, maximum (max) and minimum (min) relative abundance (%) of the Ion Torrent™ output, determined by overall cumulative abundance

Phylum	Class	Order	Family	Genus	Overall Mean	Max	Min	KGB	UR	UR	Control
Mucormycota	Mortierellomycotina	Mortierellales	Mortierellaceae	*Mortierella (parvispora)*	22.13	76.59	3.49	8.66	29.48	31.44	23.82
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	*Pseudogymnoascus*	11.32	26.33	1.20	17.83	9.35	7.74	8.58
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	*Sphaerosporella*	11.06	50.90	0.00	16.28	16.39	6.05	4.10
Mucormycota	Mortierellomycotina	Mortierellales	Mortierellaceae	*Mortierella (alpine)*	7.32	22.26	0.63	2.13	6.02	12.11	11.09
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Eupenicillium*	7.07	21.63	0.43	13.49	6.59	2.11	4.53
Ascomycota	Eurotiomycetes	Onygenales	Onygenaceae	*Chrysosporium*	3.83	32.44	0.04	0.65	1.57	8.58	3.10
Basidiomycota	Tremellomycetes	Tremellales	Trimorphomycetaceae	*Saitozyma*	3.76	16.80	0.31	10.83	0.92	0.82	1.07
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium (OTU12)*	2.92	6.91	0.45	4.61	2.02	1.96	3.34
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium (OTU14)*	2.33	6.95	0.40	1.66	3.05	1.10	4.19
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Chromocleista*	2.33	6.04	0.32	1.16	2.76	2.71	3.08
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Myxotrichum*	2.33	24.87	0.00	0.02	0.53	7.49	0.54
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	*OTU11*	2.21	8.50	0.29	1.71	2.20	1.38	3.32
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*	1.71	8.45	0.28	1.21	1.23	1.07	2.98
Mucormycota	Mortierellomycotina	Mortierellales	Mortierellaceae	*Mortierella (OTU571)*	1.64	6.25	0.04	0.16	1.27	3.26	2.43
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae,	*Polyphilus*	1.46	4.20	0.23	1.27	1.33	0.89	2.20
Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	*Hymenochaete*	1.05	6.90	0.00	2.12	0.67	0.40	0.71
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*	0.98	2.99	0.08	2.11	0.71	0.22	0.73
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	*Sebacinaceae (unc.)*	0.77	2.78	0.12	0.45	0.88	0.49	1.31
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	*Hyaloscypha (OTU20)*	0.77	3.47	0.19	0.34	1.31	0.45	1.78
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	*Hyaloscypha (OTU19)*	0.70	1.77	0.15	0.57	0.81	0.43	1.11

4.4.7. Biostimulant effects

Biostimulant treatment was found to have had a significant effect on Basidiomycota abundance (ANOVA, p = 0.01) (Figure 4.3). KGB treatments had significantly higher Basidiomycota abundance than UM or UR treatments (ANOVA, p = 0.02 for both pairs). In particular, *Saitozyma* abundance was significantly affected (ANOVA, p = 0.0001). The treatment KGB was associated with increases in *Saitozyma* abundance that were not affected by sterilising the biostimulant or by P-amendments (post-hoc Tukey; KGB-Control, p = 0.0004; UM-KGB, p = 0.0003 and UR-KGB, p = 0.0003). A combined effect of phosphorus and biostimulant treatment was found to have a significant effect on *Trichoderma* (Two-way ANOVA, p = 0.04), which was more abundant in P-amended substrate treated with UM than in the non-P amended treatments. Sterilised treatments which had no P-amendments produced only trace abundance of Trichoderma OTUs. NMDS ordination (Figure 4.8) was able to discern separation in fungal community structure between the biostimulant treatments

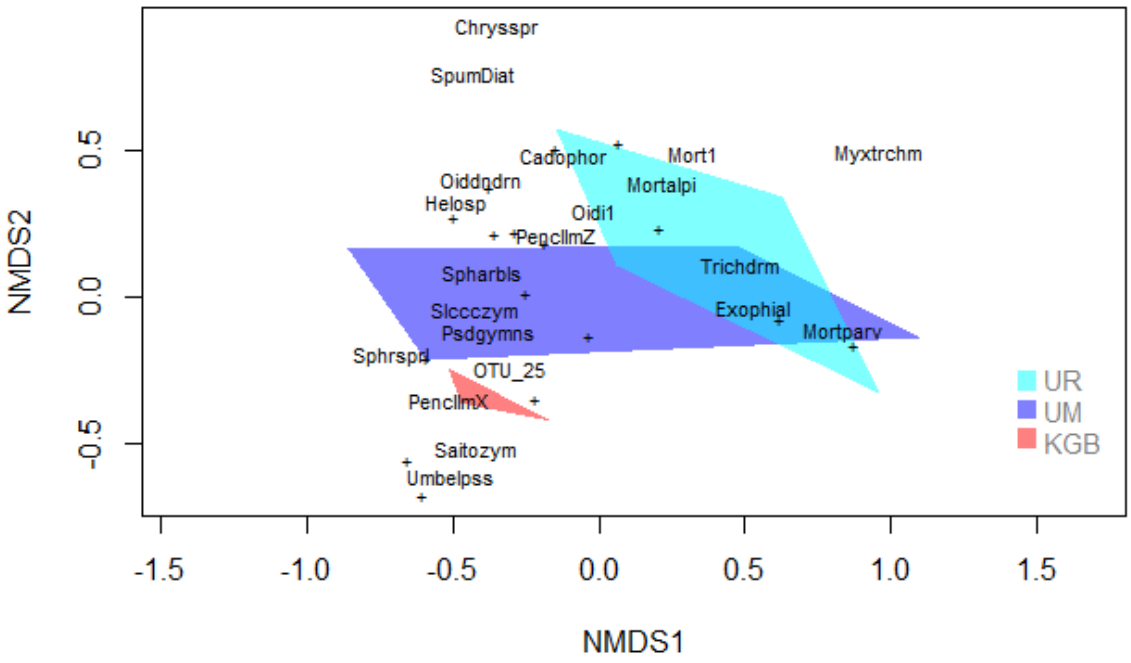

Figure 4.8: Non-metric multidimensional scaling analysis of fungal community data across all three biostimulant treatments. Some separation is apparent between convex hulls drawn between the UM and UR treatment community data, and clear separation is apparent between both and the KGB treatment community data

Figure 4.9: Stacked barchart showing mean abundance of the top eight most commonly occurring Ascomycota (a) and top five most commonly occurring Basidiomycota (b) across the three live and sterilised biostimulant treatments and controls in P-amended and non-P amended substrate. Some taxa associated with plant growth promotion are clearly represented and show considerable variation between treatments (particularly *Trichoderma, Saitozyma* and *Solicoccozyma*)

4.4.8. Phosphorus effects

Phosphorus amendments were found to have an overall significant effect on fungal community taxa abundance (MANOVA, p = 0.008), and principle coordinates analyses of fungal community structure was able to discern clear separation (Figure 4.10). In order to identify which fungal species and which fungal functional groupings were most affected by different P-amendments and whether this varied depending on live or sterilised biostimulant treatments, the relative proportions of the top 30 taxa were analysed for variance. Phosphorus amendments were significantly associated with an increase in the abundance of the overall most abundant taxon *Mortierella parvispora* (ANOVA, p = 0.005). P-amendments were also significantly associated with increases in the abundance of *Chromocleista* (ANOVA, p = 0.0005) that were not associated with biostimulant treatment or whether the treatment was live or sterilised (Tukey, post hoc). P-amendments had a significant effect on Mucormycota taxa abundance (ANOVA, p = 0.0005), which largely consisted of species from the *Mortierella* genus. Ascomycota abundance was significantly reduced by P-amendments (ANOVA, p = 0.04), although the Ascomycota community structure of KGB treated substrate remained relatively consistent under all treatments.

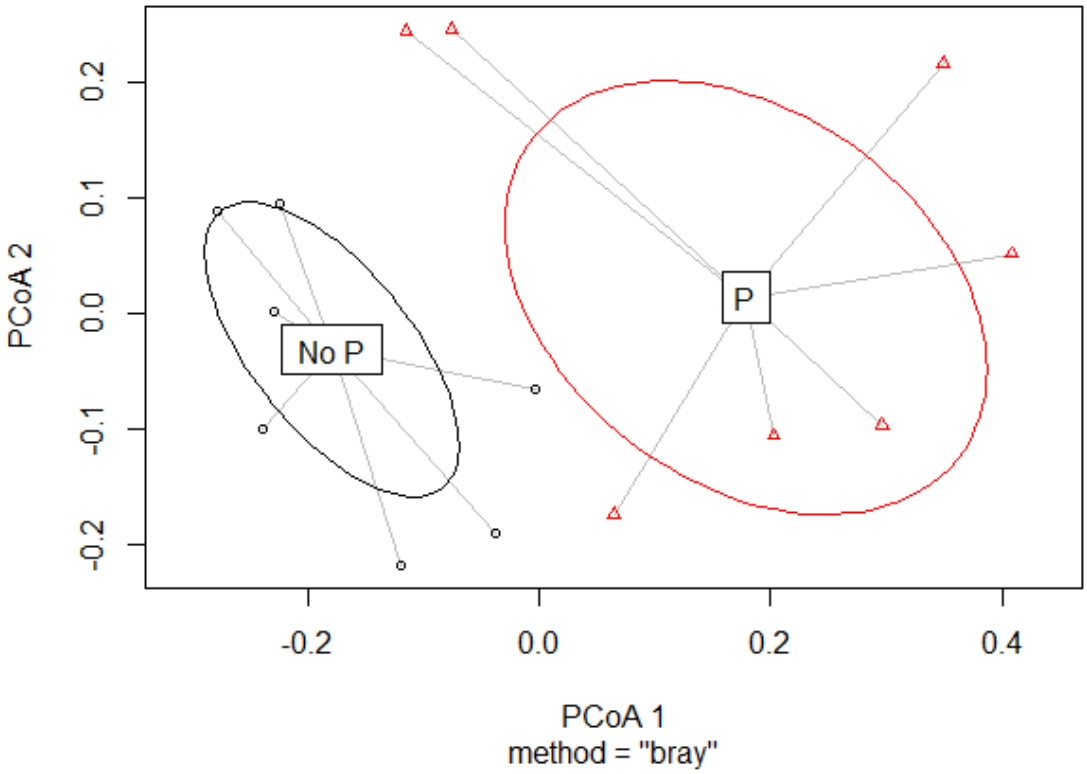

Figure 4.10: Principal Coordinates Analysis of fungal community data across all samples showing P-amended treatments ("P) and no added P ("No P") Ellipses represent a 95 % confidence interval, separation between the two treatments is clearly apparent

4.4.9. Ecology

Both biostimulant treatment and P-amendments were found to affect Inverse Simpson index (Two-way ANOVA, p = 0.008 and p = 0.002 respectively). All biostimulants in both P- amended and non-P amended treatments had significantly lower inverse Simpson index values than the control (Table 4.5). P-amendments were also associated with significantly reduced Shannon index values (ANOVA, p = 0.05), and also significantly reduced overall fungal taxa count (ANOVA, p = 0.01) and OTU count (ANOVA, p = 0.003). Sterilising the biostimulant was associated with a significantly reduced total number of fungi in treated substrate (ANOVA p = 0.05).

Table 4.5: Differences in Inverse Simpson index values between biostimulant treatments and controls (no added P) which had a mean inverse Simpson Index of 21.21. Significance codes: 0.001 **, 0.01 *. Effects of biostimulant sterilisation on Inverse Simpson Index were not significant and are therefore not reported.

Treatment	Inverse Simpson Index	Significance
KGB, no added P	9.81	0.05 *
UM, no added P	7.01	0.02 *
UR, no added P	5.66	0.01 *
KGB, P-amended	7.92	0.03 *
UM, P-amended	3.69	0.008 **
UR, P-amended	4.12	0.009 **

Figure 4.11 summarises mean abundance of the broad ecological groupings associated with the fungal taxa identified. Biostimulant treatment and P-amendments had a significant combined effect on Dark Septate endophyte abundance (Two-way ANOVA, p = 0.04), with significantly reduced abundance of DSEs compared to the controls (Tukey post hoc, p <0.05). Saprophytic fungi abundance significantly increased with P-amendments (ANOVA, p = 0.006).

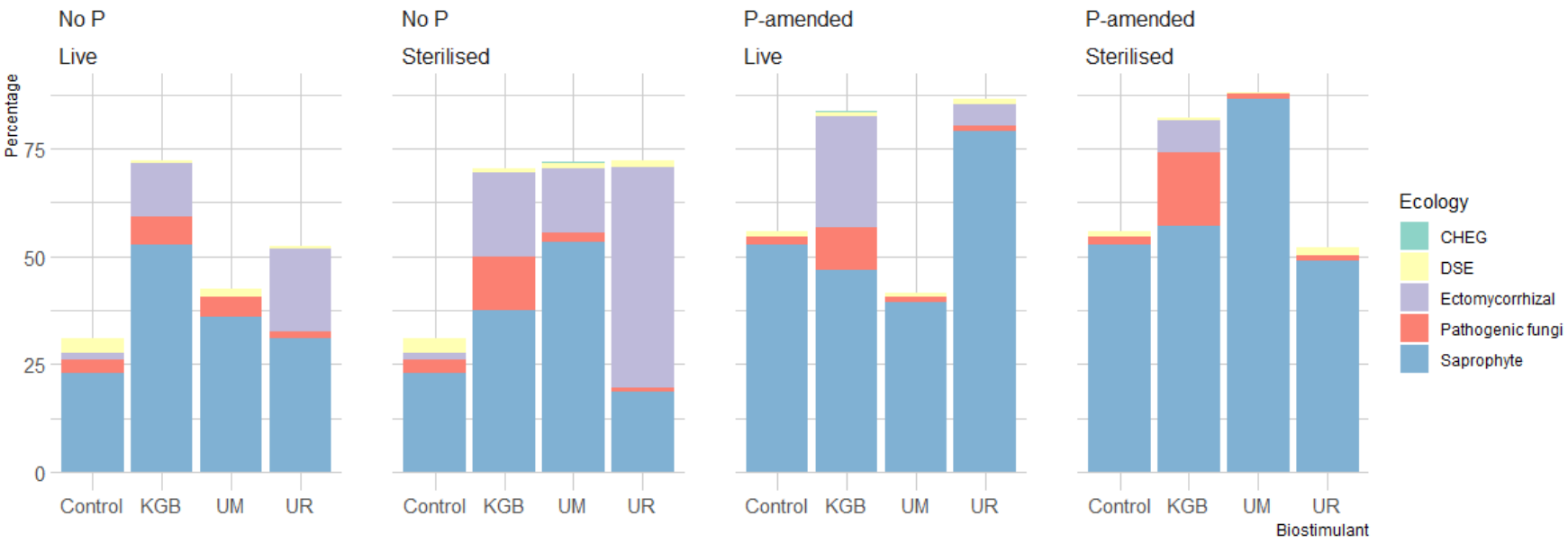

Figure 4.11: Stacked bar chart showing mean abundance of the five identified ecological groupings across the three live and sterilised biostimulant treatments and controls in P-amended and non-P amended substrate ("CHEG" = *Clavaria Hygrocybe Entoloma Geoglossum* (grassland fungi), "DSE" = Dark Septate Endophytes).

4.5. Discussion

Unlike Chapter 3, there was lower leaf P concentrations in plants receiving biostimulant treatments than in the P-amended control, which could be evidence of a cost to the plants as a result of certain properties of the biostimulant. This could be a direct, metabolic cost due to a symbiosis drawing carbohydrates from the plant but not offering any physiological benefits in return (Berdeni *et al*., 2018), or there could be some interference with crop P uptake pathways (Romera *et al*., 2019). Regardless of the mechanism, the findings of this study suggest that even in P-limited soils, biostimulants may not be able to improve crop phosphorus uptake, even though they may be able to improve yield or improve growth morphology. Although there is potentially some weak evidence that the live biostimulants may be able to increase crop P uptake compared to the sterilised controls, thereby suggesting that carrier effects may not entirely account for observed improvements, the fact that the leaf tissues of the control treatment had higher P levels than the biostimulant treated plants suggests that in the experimental conditions used in this study, biostimulants were not an effective means of improving crop phosphorus uptake.

As discussed in Chapter 3, some discrepancy was observed between the manual measurements and the PSI system data which resulted from the sideways positioning of the camera in the method used in Chapter 3. In this study, the use of *Arabidopsis* rather than *Lolium* has meant that a rosette has been measured via a camera positioned above the plants, which has ensured only pixels from plants in their own pot have been recorded, rather than accidentally recording pixels from other plants drooping into their growing space. This has allowed the method to maintain the benefits of a 2D analysis system while addressing the issues of a sideways-positioned camera. Diverging from the evidence presented in Chapter 3, the Olsen P method did not suggest that, in the case of *Arabidopsis*, improved phosphate availability accounted for much of the differences observed between treatments. At the end of the study, all but three treatments had resulted in relatively similar levels of plant-available P. However, the P amendments were associated with significantly reduced biomass in all treatments in contrast to non-amended treatments (biomass inferred from rosette area; Duan *et al*., 2018; Kaushal *et al*., 2016), and two of the sterilised biostimulants in non-P amended soils produced the highest overall growth (area and perimeter). This would suggest that the elemental composition of the biostimulant (in this case DAP and zeolite) is largely associated with any improvements to plant growth observed at the scales and application rates used in this trial. To this extent, the work undertaken in this chapter supports the findings of Chapter 3, where it was observed that the sterilised biostimulants performed comparably to the live biostimulants in terms of improved growth in the trial plants.

In non-P amended treatments, live biostimulants generally performed less effectively than sterilised biostimulants in the regard of improved plant growth-promotion. Elemental analysis of the sterilised biostimulants confirmed that there were no significant changes to chemical composition after sterilising. This would suggest that in the experimental conditions in this study, the microorganisms introduced by the biostimulants negatively affected plant growth morphology (Berdeni *et al*., 2018; Romera *et al*., 2019). However, this does not mean that the live biostimulants are unable to afford plant growth-promoting benefits in other soil conditions with different microbial community structures and different abiotic conditions (Awasthi, 2019; Santini *et al*., 2021). This is demonstrated by the live UR treatment plants outperforming other treatments in P-amended condition. These findings would support the general conclusion that no individual biostimulant will improve crop growth under all conditions. In this case, since the live UR biostimulant was associated with improved plant growth in P-amended conditions, it could be possible that microbial community shifts associated with improved plant growth-promotion are detectable. This would necessitate further research into changes in microbial community structure associated with beneficial changes in plant growth and morphology after biostimulant or nutrient amendments.

For the *Arabidopsis* growth assessment, rosette growth was analysed for a period of 40 days. Rosette development normally follows a sigmoid pattern with a lag phase, which is usually displayed by a 10 day period of slow growth, followed by a period of acceleration, and then slowing when the transition from the vegetative to the reproductive phase is reached (Pavicic *et al*., 2017). Neilson *et al*. (2015) assert that sigmoid behaviour is best modelled by a three parameter logistic regression (3PL) which describes these three stages. However, a 3PL model was not suitable for the data collected from this method due to the unconventional behaviour of some of the treated plants (UM and UR in particular, which are to some extent the key focus of this study). While polynomials could have afforded greater flexibility (Pavicic *et al*., 2017), local regression afforded greater flexibility still and offered more explanation of the data for all the parameters. This was of particular importance in the case of the complex parameters of roundness, roundness 2, isotropy, compactness and RMS. Roundness values typically decay over time as a result of rosette perimeter increasing at the same time as leaf proliferation, which was generally observed across all treatments. Faster decays in roundness were associated with more vigorous growth, particularly in the case of sterilised UR in non-P amended soils. For crops destined for harvest, the transition from the vegetative to the reproductive phase should be made early to allow ripening and avoid abiotic stress (Camargo *et al*., 2016), and the more rapid decay in roundness shown by the live and sterilised UR treatments in non-P amended soils and the live UM treatment in P amended soils could be an indicator of this transition. However, in P-amended soils, it was only the rhizobia (UR)

biostimulant that was associated with higher rosette area than the other treatments and the controls, and therefore it is unlikely that the circularity changes associated with the mycorrhizal (UM) treatment would have translated into any yield increase.

The number of variables associated with rosette measurement are considerably greater than those collected for *Lolium* leaves in Chapter 3. Circularity, symmetry and centre distance have all been quantified in considerably more depth than in Chapter 3. Usually, the parameters of roundness 2, isotropy and RMS will increase and decrease over time, which results from the cycle of leaf initiation and expansion (Pavicic *et al*., 2017). Rosettes will begin this cycle with an elliptical shape which develops into a more circular one when the third and fourth leaves begin to develop. Since these leaves will continue to develop after the first two leaves have ceased to grow, an elliptical tendency will then be observed again in the second week. The repetition of this cycle explains the oscillatory nature of the circularity parameters, and may suggest the value of polynomial models or localised regression to visualise this data as opposed to other models (Pavicic *et al*., 2017).

Morphometric data from RGB analysis suggests that two biostimulant treatments were associated with some significant growth improvements in *Arabidopsis*, these treatments being UM and UR, and all data collection methods recorded that the autoclaved UM and UR biostimulant treatments (UM(S) and UR(S)) produced growth results exceeding those of their non-sterilised counterparts However, and crucially, these treatments were not associated with improved P concentrations in the plant tissues. These findings support the suggestion made in Chapter 3 that the microorganisms present within the UM and UR biostimulants did not necessarily contribute to the enhanced growth observed on plants treated with it. The exception to this observation is in the case of live UR applied to P-amended soils, which produced a significantly greater area curve than all other treatments. However, this area was still considerably less than the growth observed in non-P amended soils. This may be a result of the combination of P introduced from the biostimulant and the P-amendment producing an overabundance of P, although the plant available P assay demonstrated that the majority of treatments had comparable P levels. It is therefore likely that the excess P that was not measured in leaf tissue was leached from the treated soils. Root P levels were not measured in this study, and whilst a proportion of total plant tissue P is anticipated to be present in the roots, plants typically prioritise supplying P to meristematic tissues (Horst *et al*., 1996) and therefore it is unlikely that the root tissue P proportion will account for much of the unaccounted P.

In the P-amended soils displaying more growth from live biostimulants than sterilised ones, it is possible that microbial activity contributed to plant growth-promotion as a result of the

treatment. Even if this was not the case that microorganisms were actively introduced through the biostimulant which aided plant growth, the treatment could still have stimulated microbial biomass growth through improved rhizosphere equitability to plant growth-promoting microorganisms. Further discussion of this is presented in Chapter 3. Further research would appear necessary to determine the specific factors that improve rhizosphere equitability for plant growth-promoting microorganisms.

The potting substrate used in this trial consisted of sterilised compost (as in Chapter 3). This has a number of important implications. Firstly, this prevents any discussion from these findings as to the influence of priority effects, interactions between resident microbial communities in soil and introduced microbes from biostimulants, or metabolic niche overlap (Parnell *et al*., 2015; Kaminsky *et al*., 2019). Secondly, sterilised compost may result in greater nutrient availability and enhanced plant mineral nutrition than in natural soil, because the microbial biomass will no longer be competing with plants for nutrients, and sterilised microbial biomass will itself become a source of mineral nutrition for plants (Yang *et al*., 2015; Hu *et al*., 2019). This is one reason for the large discrepancies between laboratory and field trials in terms of reporting biostimulant efficacy, which may be greatly exaggerated in the absence of native microbial populations. This raises additional questions for biostimulant manufacturers who address persistence and degradability questions by claiming that biostimulant microbes are naturally present in the environment (Symbio, 2019). If this is the case, it is unclear how the biostimulant is intended to add value to the soil microbial community, especially if the evidence base for microbial species selection rests largely on evidence from lab trials (Kaminsky *et al*., 2019). As described above and as demonstrated in this study, many lab trials explore microbial effects in a much more sterile environment than in agricultural conditions (Rouphael *et al*., 2015), and it does not make sense to use evidence of biostimulant performance in a sterilised environment to justify introducing microbes into agricultural environments that are apparently already present. The seeds used in this trial were not surface sterilised, which was on account of the fact that seeds are not typically sterilised before sowing in agricultural contexts. However, the absence of data concerning resident seed microbial data is a caveat for interpreting this data, as it prevents discussion of how microbes resident in the seeds might interact with the biostimulant or the potting substrate.

An issue which may affect all plant trials of this kind is that promotion of plants in soil as a consequence of plant growth-promoting microorganism inoculation may not necessarily be associated with P solubilisation which are manifest under laboratory conditions (Sanchez-Evesta, 2016). It is possible to examine the role of fungal biomass in plant growth-promotion carrying out an ergosterol assay (which is carried out on the soils used in this experiment in Appendix 3), although this method is not able to discern AMF biomass (Olsson *et al*., 2003).

Since a non-mycorrhizal experimental crop was used in this study, ergosterol may actually have potential to add value to this method, by contextualising diversity measurements with biomass measurements.

Other key variables that would elucidate biostimulant effects on plant growth and health would be water use, respiration and photosynthetic efficiency (Quilliam *et al*., 2006). Given the substantial differences detected between growth rates across treatments in this study, these measurements may offer insights as a combined effect with nutrient use efficiency, and such work requires further investigation, possibly by using full spectrum and infrared reflectance/absorbance, which would ideally be taken continually throughout the growth period rather than at a single time point as was the case for the chlorophyll fluorescence measurements presented in Chapter 3.

In addition, only one ecotype (Col-0) was used in this study, whereas future research should consider different plant origins. The PSI system was able to provide useful information which corresponded accurately with manual measurements, considerably more so than with the method used in Chapter 3. In Chapter 3, some further shortcomings inherent with 2D imaging has which affects the accuracy of the information it can generate is discussed. Although the approach used in this chapter has addressed some of these issues, the limitations still remain that 2D imaging cannot collect volumetric data and is limited in its collection of spatial data compared to 3D imaging (Duan *et al*., 2018). However, *Arabidopsis* growth can be characterised fairly accurately by analysing the rosette development as described in this study. Further discussion of the trade-off between accuracy and detail in 3D plant imaging is presented in Chapter 3. The chief limitation remains the lack of capacity for high-throughput analysis offered by 3D analysis.

This also study set out to determine whether different agricultural biostimulants and phosphorus amendments affected soil fungal populations. PCoA and NMDS analysis showed that the fungal community structure clearly separated after biostimulant and P-amendments, which can also be associated with the changes in growth morphology seen in the *Arabidopsis* plants after P-amendments and biostimulant application. Morphometric data suggests that the UM biostimulant treatment (a consortia of plant growth-promoting fungi, DAP and zeolite carrier) was associated with larger rosette areas and steeper growth curves than controls and other biostimulants in P-amended soils, and that the live UM biostimulant treatment produced greater rosette areas than when sterilised, although this was not associated with improved P uptake in plant tissues. However, in non-P amended soils, the UM and UR biostimulant treatments produced significantly less growth than their sterilised counterparts.

Before interpreting the findings of this study, it is important to note that the experimental substrate was not representative of agricultural soils. The experimental pots used in this study do not replicate physical or chemical properties of agricultural bulk soil, nor do they replicate typical microbial community structure, as evidenced by the lack of CHEG fungi, for example. This grouping, consisting mainly of basidiomycetes and including members of the families Clavariaceae, Hygrophoraceae, Entolomataceae and Geoglossaceae, are recognised as predominating grassland and agricultural habitats (Clasen *et al*., 2020). These fungi are obligate root-associated biotrophs, and given the relatively short experimental time period it is not unexpected that this grouping did not establish themselves in the experimental soil (Halbwachs *et al.*, 2013; Griffith *et al*., 2019), but it serves to demonstrate how different the conditions of the experimental substrate from agricultural soils.

In this study, it was observed that live biostimulants had significantly higher taxa counts than sterilised counterparts, which suggests that the biostimulant treatments tested herein promote a proliferation of fungal taxa in the soils which continue to have effects at least two months after biostimulant application. This demonstrates that the method of biofixing microbes to the carrier substrate probably was to some degree successful in introducing and establishing microbes to the soil (Frew *et al*., 2019; Chaudhary and Shukla, 2019). However, this does not allow conclusions to be made about persistence or abundance, as the diversity of sequences is not directly related to abundance, nor is two months long enough for most crops to come to harvest (Camargo et al., 2016; Munns et al., 2010), and the experimental substrate was not representative of agricultural soils (Halbwachs et al., 2018; Clasen et al., 2020). Another issue and particularly the case with bacteria is that microbes can replicate many times between the biostimulant refinement stage and the application stage. Potentially many generations may elapse, which if populations have high degrees of genetic variability could lead to different functionality in the soil compared to the purported effects (Kaminsky et al., 2019). The aims of this study relate to measuring the purported plant growth promoting effects of fungi, but rhizobia biostimulants were also used, and under some experimental conditions performed more effectively than the mycorrhizal. Therefore, it would have been very useful to know more about how bacterial diversity was affected by the treatments in this study. Repeating the process with different primers would be able to obtain such data (Hartmann *et al*., 2014).

All biostimulants in both P- amended and non-P amended treatments had significantly lower Simpson diversity index values than the control, and P-amendments were also associated with reduced Shannon index values, the number of fungal taxa and OTU count. This could suggest that biostimulants can affect microbial community structure and soil equitability whether or not changes in crop growth are observable. Some live biostimulants (UR, containing DAP and zeolite) was associated with higher growth rates in elevated P, but its

sterilised counterparts produced significantly greater growth rates in non-P treated soils. In non-P amended soils, UR treatments had the lowest mean inverse Simpson index, but it is not possible to determine whether this has any connection to improved equitability for plant growth-promoting species.

The findings of this research suggest that the products investigated can significantly affect fungal community structure whether or not they have been sterilised, which was one of the main areas noted for further investigation in Chapter 3. *Saitozyma* abundance was significantly associated with KGB (BrassiKa) treatments, and in KGB-treated soils it remained relatively constant regardless of P-amendments or whether the biostimulant had been sterilised. Elemental analysis showed that KGB carrier composition consisted of 35 % carbon, whereas other biostimulants were all below 0.3 % C. These results may indicate that a carbon rhizosphere priming effect (Adamczyk *et al*., 2019) may be associated with *Saitozyma* abundance, but further research would be needed to corroborate this. Significant production of IAA (>1000 µg/g) is associated with the yeasts *Saitozyma* and *Solicoccozyma* genera (formerly *Cryptococcus*) as described in Streletskii *et al*. (2016). These effects are detectable in vitro, but field scale studies which account for this are lacking (Yurkov, 2018). Yurkov (2018) also describes *Saitozyma* diversity as a possible indicator of soil equitability associated with well-drained acid soils, but as explained above, due to the experimental substrate lacking key biological similarities to agricultural soils, equitability indications cannot be made from these findings, which would require similar patterns to be observed in field studies under similar treatments (Lentendu *et al*., 2014; Hartmann *et al*., 2014).

A genus which displayed significant shifts in abundance associated with phosphorus was *Mortierella*. Other studies have documented reductions in *Mortierella* abundance after N application (Arnebrant, *et al*., 1990), the authors attributing the changes to difference in pH. A number of studies document the importance of pH as a determining variable of fungal community structure (Tedersoo *et al*., 2014), and since this study sought to restrict determining variables responsible for shifts in fungal community structure to P-amendments and biostimulant treatment, care was taken to ensure homogeneity of pH in the soil substrate (see Chapter 3). In contrast, Detheridge *et al*. (2016) documented positive correlations between *Mortierella* and soil N levels, and a negative correlation with soil P levels, although Detheridge et al. (2018) were examining changes in fungal community behaviour in field conditions. In the present study, it was observed that in P-amended treatments, the genus *Mortierella* constituted the majority of fungal taxa observed in the live UR-treated soil, and was also the most abundant genus in the soil treated with sterilised UM. Both treatments produced the largest growth curves in their respective conditions, which would suggest a possible role of *Mortierella* in improved P-use efficiency. Detheridge *et al*. (2016) propose that *Mortierella* may

be actively involved in P mobilisation from inorganic sources by organic acid secretion, which can chelate bound cations (see Chapter 1 for more discussion of this mechanism). Neither *Mortierella* nor *Saitozyma* are likely to have endophytic/mycorrhizal modes of action, as Nallanchakravarthula *et al*. (2014) were able to demonstrate by showing that neither genus were present in root tissue but were the most abundant taxa in the rhizosphere. In pot trials similar to this study, *Mortierella* has been associated with increased plant P uptake, and may act synergistically with arbuscular mycorrhizals (Osorio and Habte, 2013). However in this study, there were no obvious associations between *Mortierella* relative abundance and improved crop P uptake, other than that the genus was more abundant in soils which had higher plant available P. In any case, the behaviour of *Mortierella* in this study was not being studied in a representative agricultural soil (Lentendu *et al*., 2014; Hartmann *et al*., 2014), and therefore more research in field environments will be necessary to quantify whether the possible mode of action described above can influence crop yield.

In this study, a combined effect of the UM biostimulant and P-amendments were found to increase *Trichoderma* abundance. This species is a well-documented plant growth-promotor, and is a common species used in biostimulants. Stewart and Hill (2014) describe its effects in detail, which include increased root and shoot biomass as well as improved morphology, which are likely a result of improved hormone balance with respect to IAA, ethylene and gibberellic acid. An uncertainty often reported with biostimulants relates to inconsistency and viability of batches (see a more detailed discussion of this in Chapter 2), and this study has demonstrated viability of *Trichoderma* in treated soils at least two months post-inoculation (sterilising the biostimulant resulted in only trace abundance of *Trichoderma*). Again, these findings do not account for the competition for nutrients in agricultural soil that introduced microbes will face (Romera *et al*., 2019), so further work is necessary to determine whether hormone-balancing effects or pathogen suppression effects (often attributed to *Trichoderma;* Berdeni *et al*. (2018)) are observable months after inoculation, and therefore this kind field experiments will be necessary to corroborate these findings. It was not possible within this study to discern if the increased abundance of *Trichoderma* was of native or applied origin, as *Trichoderma* can be readily found in the environment and it is possible that come airborne contamination through the facility ventilation system could have introduced *Trichoderma* spores to the experimental substrate (Symbio, 2019). Future methodologies may be able to address this by culturing *Trichoderma* isolated from the biostimulant and contrasting genotypes with *Trichoderma* sequenced from the treated soils (Mukherjee *et al*., 2013), or experimental procedures could include operating in even more sterile environments with purified ventilation to prevent airborne spore ingress. This would of course be even less representative of agricultural soils and would restrict the degree to which interpretation could be made relating to purported

microbial modes of action, but it would clarify the extent to which cross-contamination could have led to the establishment of supposedly plant growth-promoting microbes that have been claimed to have been introduced to soils by biostimulants (Hart et al., 2018). In this study, the background microbial count and composition of the microbial communities living on the seeds were not examined, which could include species which later established in the experimental substrate. Attempts were made to investigate the microbial composition of the biostimulants before they were introduced to the soil to confirm the manufacturer's information on species composition presented in Table 4.1. Unfortunately, neither ordinary molecular diagnostic nor PowerSoil extraction methods obtained results, and separate work should be undertaken to verify the manufacturer supplied information on species composition, which could be misleading for various reasons (Hart et al., 2018 Kaminsky et al., 2019).

Sterilised biostimulants in non-P amended soils had a significantly higher abundance of ectomycorrhizal fungal taxa, and this treatment pairing was associated with the largest overall plant area during the growing period, although not the highest levels of crop P uptake. In particular, sterilised UR treatments were associated with the largest number of ectomycorrhizal fungi in non-P treated soils (mainly consisting of the genus *Sphaerosporella*). As the biostimulants were sterilised, they could not directly have introduced ectomycorrhizal fungi to the rhizosphere but they may have contributed to improved equitability for ectomycorrhizal species, possibly through zeolite-mediated effects which have elsewhere been documented to improve P-use efficiency in plant tissues (Ahmed *et al*., 2010). Ectomycorrhizal fungi usually form symbioses with woody plants, but recently Detheridge *et al.* (2016) have suggested that some ectomycorrhizal fungi may form mutualisms or endophytic associations with non-woody host species in agricultural contexts. The present study supports this finding and would suggest that it should be subject to further research, given that the experimental substrate was not representative of agricultural soils, as explained earlier.

The NGS technology used in this study could be used in conjunction with other platforms to analyse microbial activity. A sterols assay is one such method, which could be used to quantify fungal biomass (Wallander *et al*. 2013). Ergosterol is present in most fungal membranes, although not in AMF (Olsson *et al*., 2003). Due to the rapidity of the decomposition of membrane components after fungal cell death, ergosterol can provide reasonable estimates of living fungal biomass (Newell, 2001). In this instance, it would be useful to be able to correlate taxa abundance with fungal biomass, as this could afford greater insights into the effect of biostimulants on diversity of the microbial community structure post-inoculation. Since AMF were not expected to form symbioses in this study due to the experimental crop being non-mycorrhizal, and there was almost no evidence of any AMF taxa in this experiment, a

sterols assay could be useful to offer another measure of fungal change associated with biostimulants to contextualise community structure data.

Only one time point was measured using this experimental method, which could have missed crucial information about priming and priority effects (Hart et al., 2018). The method used in this study could be expanded to examine initial priming effects of biostimulants by taking DNA samples of the soil substrate immediately post-inoculation, and then again two weeks into the growth period when transition from the vegetative to the reproductive phase is reached (Pavicic *et al*., 2017). Sampling soil substrate treated with biostimulants in the longer-term could help to explore the legacy effects of biostimulants, as the experimental period was not long enough to make conclusions about persistence.

4.6. Conclusion

The method used in this study was able to document differences in growth rates and a comprehensive range of morphological parameters of an *Arabidopsis* crop treated with different biostimulant and phosphorus forms. This provided detailed insights into the responses of the experimental crop to the effects of the treatments used. The study suggested that under elevated P, some of the live biostimulants examined herein were more efficacious in improving plant growth and morphology than under reduced P, but that treatments were not actually associated with improved crop P uptake. The findings also suggest that some supposedly plant growth-promoting microbial cultures actually stimulate negative responses from the plant in certain conditions, indicating a probable metabolic cost to the plant host. This requires further research to examine what shifts in community structure occur after biostimulant treatments have been applied, and requires contextualising in representative agricultural soils.

4.7. References

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Chapter 5

The effects of sustainable intensification approaches on fungal community structure in an upland pasture

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5.1. Abstract

The use of rotational grazing and targeted nutrient applications in upland grasslands are advocated as approaches which can increase agricultural productivity without maintaining, or even decreasing inputs, which is an inherent principle of “sustainable intensification”. The impact of these approaches on upland microbial populations has not been assessed. Soil fungal communities directly affect soil processes through various means, and are particularly important in the context of plant mineral nutrition. While these mechanisms have been well studied in pot trials, there is a shortage of evidence which examines the effect of these management approaches on soil fungal populations in field experiments, which is particularly important given the additional biotic and abiotic influencers in field environments that are not accounted for in laboratory environments. In particular, mycorrhizal species, which are well associated with improved plant nutrition are largely overlooked when agricultural management approaches are implemented. In this study, DNA metabarcoding (Ion Torrent) was undertaken using high-throughput sequencing of the D1 region of the large ribosomal subunit to assess the effect of grazing regimes and sustainable intensification methods on soil fungal population structures. The study comprised four treatments on an upland experimental site grazed with sheep: set-stocked with no nutrient amendments; set-stocked with targeted lime, phosphate (P) and potassium (K) inputs; rotationally grazed with no nutrient amendments; and rotationally grazed with targeted lime, P and K inputs. Treatments were found to have significant effects on fungal community structure, with increased relative abundance of arbuscular mycorrhizal fungi (AMF) in rotationally grazed treatments which had received nutrient amendments. Some AMF taxa including *Claroideoglomus* and *Paraglomus* showed significant increases in relative abundance after nutrient amendments, while other AMF genera showed significant decreases, including *Ambispora.* Nutrient amendments were also shown to increase the relative abundance of a number of genera which contain economically important grass and sedge pathogens, particularly *Kriegeria* but also including *Cochliobolus, Entyloma,* and *Physoderma*. Taxa counts and diversity indexes were highest in samples obtained from the set-stocked treatment which had no nutrient amendments, which suggested that grazing and nutrient regimes which increase soil N-availability in upland grasslands will be associated with reductions in diversity in fungal populations in the soils. Basidiomycota were found to dominate upland soil fungal community structure, constituting ca. 80 % of sequences across all treatments. As sustainable intensification is likely to be a major component of future management of the uplands, it is important that the development of biostimulants prioritises ensuring persistence and establishment in these environments, if they are to be used at all.

5.2. Introduction

Upland grasslands are essential for delivering of a range of ecosystem services, which include storage of water and carbon, timber production, and conservation of biodiversity. Alongside this, uplands are used for the production of livestock, which in UK constitutes the vast majority of economic activity derived from uplands, and directly supports rural communities and economies. A number of restrictions are often in effect in upland agricultural systems which limits their potential for efficient food production, including low pH, altitude, and exposure to harsher climates (Grosso *et al*., 2016; Gibbons *et al*. 2014; Wilberforce *et al*., 2003). Sustainable intensification practices are necessitated by the imperative to safeguard global food security, which requires more efficient use of existing land used in agriculture. In upland ruminant systems, the lack of targeted nutrient management has been linked to reduced grass production (Gibbons *et al*. 2014). However, efforts to redress this has in recent years been the subject of major policy initiatives, including Defra's Sustainable Intensification Research Platform (Defra, 2018).

In the lowlands, sustainable intensification practices including zero tillage, direct seeding and crop diversification have been found to improve soil quality and increase yields, with fungal population density recognised as a key quality indicator of soil health and function (Choudhary *et al*., 2018). It has now been documented that plants can manipulate their rhizosphere microbiome, with evidence consisting of different plant species hosting specific microbial populations under the same soil conditions (Berendsen *et al*., 2012), and the fact that analysis of crop root microbial communities have been shown to have lower species richness and a higher abundance of specific taxa than in bulk soil (Wang *et al*., 2018). To achieve sustainable land use in agriculture, an understanding of the dynamics of the interactions between crops, soil and microbial communities is increasingly important, and in particular the role of soil microbial populations in plant growth-promotion and how agricultural management approaches impact these communities and affect soil function (Detheridge *et al*., 2016). These interaction dynamics between soil microbial populations and crops are now understood to have comparable effects to those of abiotic variables (Wubs *et al*., 2019; Wilberforce *et al*., 2003). The diversity of plant root-associating microbes is very large, generally understood to be in the order of tens of thousands of species (Berendsen *et al*., 2012), and is of vital importance for plant health. Soil productivity has been shown to be directly related to microbial community composition (Wang *et al*., 2018), as well as the abundance of several specific fungal taxa (Detheridge *et al*., 2016). While research has repeatedly demonstrated that soil productivity is greatly influenced by microbial population activity, it is not well understood how community structure, diversity and microbial biomass influence soil functionality (Wang *et al*., 2018).

The relationship between fungi and crops is complex, and depends on the ecological function of the fungi. Arbuscular mycorrhizal fungi are in particular associated with improved plant mineral nutrition, soil carbon dynamics and crop productivity (Quilliam *et al*., 2010), and as such the presence and relative abundance of arbuscular mycorrhizal species in the soil is of considerable importance. In addition, other ecological groupings may also contribute to crop growth-promotion which are less studied, and in particular this includes root endophytic fungi, the role of which in crop growth remains largely unexplored (Burns *et al*., 2015).

It is now well understood that soil pH has a very strong influence on the microbial community structure. This is because pH directly influences a number of factors, including nutrient availability and solubility, and also microbial biomass composition (Grosso *et al*., 2016; Rousk *et al*., 2009, Rousk *et al*., 2010). Changes in soil pH are also well understood to affect the fungal/bacterial ratio (Martin *et al*., 2012), which is usually reported as decreasing with increasing pH (Bååth and Anderson, 2003), which will consequentially affect the functionality of the soil microbial community. This is particular the case with AMF, the proliferation and diversity of which in the soil are understood to be controlled by soil pH and P availability (Sommerfeld *et al*., 2013).This is important, as sustainable intensification approaches in uplands will often involve addressing sub-optimal pH levels by lime additions, and the extent to which microbial community structure might respond to such approaches remains relatively unknown.

Set-stocked livestock systems are where livestock are left in the same area for relatively long periods, continuously grazing regrowth. Rotational grazing, on the other hand, is the practise of grazing land at a higher density for a short period of time (no more than three days), followed by a period of no grazing for approximately three weeks for the grass to recover; which results in increases in the yield of grass (AHDB, undated). The effects of rotational grazing in livestock production on soil ecology are well-understood (Saunders *et al*., 2010; Wilberforce *et al*., 2003), with increased intensity of manure and ground compaction influencing rates at which nutrients enter the soil and macropore structure, resulting in lower fungal/bacterial ratios and lower diversities of AMF under grazed treatments (Oates *et al*., 2012). Through such means, the short term intensified effect of livestock presence in rotational grazing is likely to increase soil fungal activity and species abundance, which benefit from the nutrient inputs in manure. However, the manner in which fungal ecology is affected by different livestock and nutrient regimes in terms of dominant species remains largely unexplored and may have several important consequences. Under medium grazing intensities, Wei *et al*. (2018) report reduced fungal biomass prior to grazing treatment, but this appears to be also be greatly influenced by nutrient addition. It has also been demonstrated that some levels of grazing disturbance is associated with increases in plant and therefore microbial diversity via increasing opportunities

for subordinate species to compete (Saunders *et al*., 2010; Martínez-García *et al*., 2018; Wilberforce *et al*., 2003). Therefore, the importance of elucidating possible effects on fungal taxa in grasslands is therefore of great significance in the decision making process of implementing sustainable intensification. In the context of biostimulants, any integrated solution that involves tailoring biostimulants to sites, or including them as a component of a broader soil improvement strategy must not only be able to establish themselves in the soil, but must also be compatible with the effects of the incumbent and anticipated nutrient management regimes used in agriculture. Even if a biostimulant can be associated with an increase in the abundance of certain taxonomic groupings, or individual taxa, there will be no actual benefit to the crops if the nutrient management regime is associated with increases in the same taxa, which would render biostimulant application unnecessary. The same applies for other purported biostimulant benefits, such as pathogen suppression or phytohormone mediation (Lugtenberg and Kamilova, 2009; Storer *et al*., 2016).

In this study, an analysis of fungal communities under four sustainable intensification treatments is presented, utilising a combination of primers which amplifies sequences from a broad range of fungi (Detheridge *et al*., 2016; Clasen *et al*; 2020; Lentendu *et al*., 2014; Hartmann *et al*., 2014). It was hypothesised that sustainable intensification methods would result in the development of diverging soil fungal populations. Rather than focus directly on biostimulant functionality, the present chapter intends to quantify plant, soil and microbial interactions in field environments under different management approaches. Improved understanding the underlying mechanisms of these interactions are essential to improving biostimulant functionality and efficacy (Calvo *et al*., 2014; Owen *et al*., 2015).

5.3. Materials and methods

5.3.1. Experimental design

The study comprised of four land use treatments established on the SIP 1 Ffridd trial at Henfaes Research Centre, Abergwyngregyn, North Wales, UK, 53.2234N,-4.0111W at ca. 250 m asl. and due south of a coniferous shelterbelt. The objective of establishing this platform was to explore ewe carrying capacity. The vegetation was categorised as a 'National Vegetation Classification MG6' grassland, which consisted primarily of *Lolium perenne*-*Cynosurus cristatus* grassland. The experimental site had received no inputs of lime or nutrients since 1982, and was stocked with sheep at low densities as part of agri-environment schemes.

A ca. 7 ha area within this site was divided into four treatments, which were: '*business as usual'* treatments with no nutrient inputs, i) rotationally grazed, (BAU RG), and ii) set-stocked (BAU SS): and the *sustainable intensification* treatments: lime, P & K inputs with a new pasture variety, iii) rotationally grazed (SI RG), and iv) lime, P & K inputs, set-stocked (SI SS) (treatments explained in Table 5.1). In the sustainable intensification (SI) treatments, lime was applied to obtain a target soil pH of 6.0. The plots undergoing sustainable intensification treatments were also seeded with high sugar grass varieties (Germinal HSG3 + Timothy mix (Germinal 2017)) two years prior to data collection. Prior to seeding, dilute glyphosate was sprayed on the SI treatments, which was for the purposes of checking the resident sward. The seed mixture used consisted of *Lolium perenne*, *Phleum pratense* and *Trifolium repens*. The seeds were applied at a recommended rate of 37 kg ha^{-1}. Stocking rates were variable depending on pasture production (as measured by a rising plate meter). Ewes were introduced or removed depending on grass cover in the plots. Soil pH was variable across the site (4.5-6.6) with the large majority being below pH 6, which is optimal for grass production (Defra, 2019). SI treatments involved a substantial application of lime (Table 5.1) to raise pH to this target, with BAU plots remaining sub-optimal. Likewise, the phosphorus status of the site was greatly varied (4.4-25.2 mg l^{-1}), with the large majority of the site below P index 2 (16-25 mg l^{-1}) recommended for grass production (Defra, 2019), and as such SI plots received substantial amendments of Triple Super Phosphate fertiliser (47 % P) to obtain target P index (Table 5.1). A similar procedure was followed for potassium, with K indexes throughout the site below target levels, with SI plots receiving Muriate of Potash applications to bring levels of K up to target (Table 5.1) (Defra, 2019).

Table 5.1: Descriptions of the treatments applied to the site, acronyms and inputs (obtained from DEFRA (2019, unp.)). Inputs show cumulative totals applied over the course of the three years preceding the experiment, followed by the respective split doses in each of those three years. After resampling at the end of year 2, it was found that lower inputs of lime, P and K would be possible in year 3 to maintain RB209 recommended levels (Defra, 2010).

Treatment	Acronym	Size (ha)	Lime (t ha^{-1})	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})
Business as usual, rotationally grazed	BAU RG	2.8 (4 x paddocks)	No additions			
Business as usual, set stocked	BAU SS	2.3				
Sustainable intensification, rotationally grazed	SI RG	3.1 (4 x paddocks)	8.2 (4.5, 3.7, 0)	150 (3 x 50)	190 (60, 120, 10)	130 (40, 80, 10)
Sustainable intensification, set stocked	SI SS	1.3				

Soil samples were taken from each plot with a 25 mm auger to a depth of 15 cm (16 cores from four 30 × 30 m sampling areas in each treatment plot, Figure 5.1) in March 2017. Soil samples were stored in a cool box while being transported off the site and transferred into a -80 °C freezer the same day. The cores were bulked into a single sample per sampling area (fresh mass approx. 500 g) giving a total of 16 samples from an original 64 samples. This was due to limited availability of space on the ION Torrent microchip at the time of study.

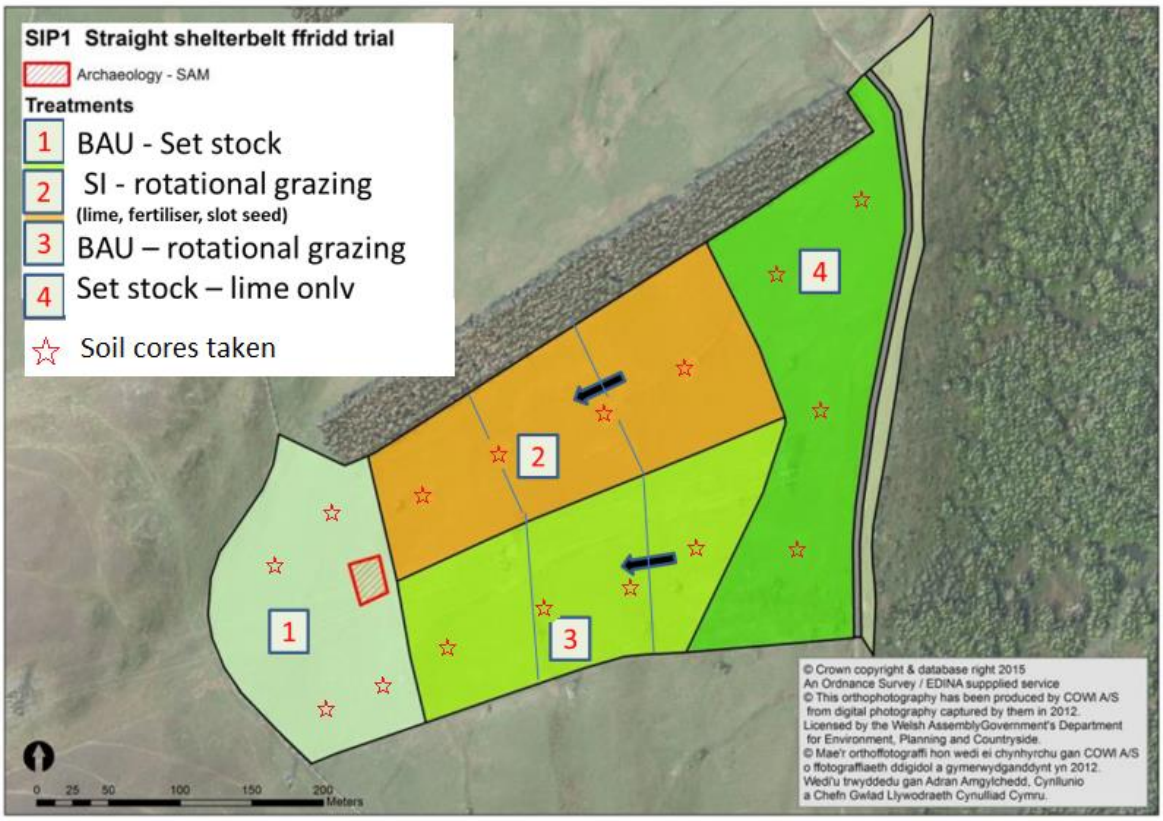

Figure 5.1: A schematic representation of the experimental site with treatments outlined. The arrow indicates the direction of grazing. Red stars denote the centres of the 30 × 30 m sampling areas where soil cores were taken (DEFRA, 2019 unp.)

5.3.2. DNA amplification and sequencing

A similar method to the process described in detail in Chapter 4 was followed for amplifying and sequencing extracted soil DNA. The soil samples from all 16 sampling areas taken in each treatment site were freeze-dried, and then frozen at -80 °C. Each sample was ground through a 500 µm sieve with care taken to ensure full homogeneity (ca. 300 g dw each) before and after grinding. DNA was extracted from 200 mg of the sieved soil sample using a PowerSoil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad USA). The D1 region of the large sub unit (LSU) of ribosomal DNA (rDNA) was amplified using fungal specific novel primers (see Chapter 4). Amplification was performed in a 50 µl Polymerase Chain Reaction (PCR) using Promega GoTaq G2 DNA polymerase (Promega, Madison USA). Each reaction contained 0.83 µl of each primer, 43.74 µl BSA, 83.48 µl dNTPs and 209 µl of DNA polymerase in the supplied buffer. Primer D1F2 was linked at the 5’ end to the IonTorrent A-adapter sequence, the TCAG key and an IonXpress Barcode. Primer NLC2AF was linked at the 5’ end to Ion Torrent B adapter sequence. The PCR conditions were 94 °C for 5 min (initial denaturation) followed by 30 cycles at 94 °C, 30 s (denaturation); 52 °C, 30 s (annealing);

72°C, 30 s (extension) and a final 5 min extension at 72 °C. PCR reactions were cleaned using spin columns (NBS Biological, Huntingdon UK) and amplified DNA were quantified using NanoDrop (NanoDrop Products, Wilmington USA). E-gel was used to purify the samples, which were then analysed and quantified using a Bioanalyser 2100 (Agilent Technologies, Santa Clara, USA). Emulsion PCR was carried out using Ion-Torrent 200 bp template kit on the Ion Torrent One Touch 2 system using the procedure supplied with the manufacturer's instructions. Amplified sequence particles were then enriched using the One Touch ES to remove non-template particles, and were then sequenced on "316" (100 Mbp) microchips using the Ion Torrent Personal Genome Machine (Life Technologies, Waltham USA).

5.3.3. Sequence data processing

A similar process was used to quality check and demultiplex sequence data as described in Chapter 4. Sequence data were quality checked and trimmed to 200 bp. Demultiplexing was performed using MOTHUR. Non-matching barcode and primer sequences were discarded. UCHIME was used to check for putative chimeric sequences against a reference database of fungal sequences downloaded from the Ribosomal Database Project (RDP) website (Detheridge *et al*., 2016). Sequences for each sample were rarefied to the lowest sequencing depth using the subsample function of MOTHUR to randomly select sequences from each file. Rarefied files were dereplicated and singletons discarded and operational taxonomic units (OTUs) were assigned using USEARCH/UPARSE (v7) at 97 % clustering, discarding clusters containing fewer than 10 sequences.

A taxonomy was assigned to each OTU (operational taxonomic unit) using the Naïve Bayesian Classifier, with a recommended cut off for 200 bp sequences of 50 %, against the RDP LSU database version 11. This ensured that all non-fungal sequences were identified at least phylum level. Where genus could not be assigned by the classifier due to low confidence threshold, an OTU identifier was assigned to that cluster. Microsoft Excel was used to render and standardise the data by dividing the number of reads in each taxonomic unit by the total number of fungal reads in each sample to provide relative abundances of assigned taxa for each sampling area; any non-fungal taxa were reported separately. Shannon and Simpson diversity indexes where P_i = relative proportion of the *i*th taxa were calculated for each sampling area (Equations 1 and 2).

Equation 1: Shannon diversity index,

$$\left(-\sum_{i=1}^{s} P_i ln P_i\right)$$

Equation 2: Simpson diversity index

$$\left(1/\sum_{i=1}^{s} P_i^2\right)$$

The broad ecological function of the fungi identified was assigned to each taxon at genus or family. If different ecological functions could be identified within a taxon, a function was only assigned when more than 75 % of known species within the taxon could be assigned to a single function. Otherwise, the function remained undetermined (Detheridge *et al*., 2016). Ecological groupings assigned were saprobic fungi (saprotrophs which decompose organic matter), pathogenic fungi (demonstrated negative effects on plant growth), AMF (arbuscular mycorrhizal fungi), EMF (ectomycorrhizal fungi), DSE (dark septate endophytes), lichens, yeasts and CHEG (families Clavariaceae, Hygrophoraceae, Entolomataceae and Geoglossaceae, which are grassland fungi which are potentially biotrophic).

5.4. Results

5.4.1 Overview

Across the 16 soil samples which were analysed, the mean number of sequences obtained after quality control was 32,824 per sample, the lowest being 21,380 (Tables 1 and 2). Of these, >96 % were fungal, the remainder being mostly belonging the genera *Cercozoa* and *Cercomonadidae* (both of kingdom Rhizaria), which accounted for 23 % and 6 % respectively of the non-fungal sequences cross all samples, and also Lophotrochozoa (kingdom Animalia), which accounted for 18.5 % of non-fungal sequences cross all samples. Basidiomycota were the most abundant fungi (mean 80 %; range 64–94 %), followed by Ascomycota (mean 12 %; range 4–23 %). More than 90 % of fungal sequences were identified to family and more than 73 % identified to genus. The most abundant genera were *Cuphophyllus* (mean 23 %; range 0.01–67 %), *Hygrocybe* (mean 5 %; range 0.12–23 %), both members of phylum Basidiomycota and family Hygrophoraceae. General statistics from sequencing run are presented in Table 5.2, and the relative abundance of the most commonly occurring taxa across all treatments is detailed in Table 5.3.

Table 5.2: Summary statistics for sequencing run

Total sequences	546365
Fungi identified to family (%)	90.93
Fungi identified to genus (%)	73.88
Ascomycota (%)	12.47
Basidiomycota (%)	80.34
Blastocladiomycota (%)	0.08
Chytridiomycota (%)	0.70
Fungi incertae sedis (%)	1.72
Glomeromycota (%)	1.61
Pseudofungi (%)	0.33
Fungi Total	32824
Non Fungi Total	1323
Inverse Simpson Index (Taxa)	9.59
Shannon Index (Taxa)	3.12
Taxa Count	302.31
Inverse Simpson Index (OTU)	10.99
OTU Count	389.81
Shannon Index (OTU)	3.42

Table 5.3: Top 20 fungal taxa across all treatments, ecological grouping, mean, maximum (max) and minimum (min) relative abundance (%) of the Ion Torrent™ output, determined by overall cumulative abundance

Phylum	Class	Order	Family	Genus	Ecology	Mean	Max	Min	BAU SS	SI RG	BAU RG	SI SS
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	*OTU 1 (Tricholomataceae)*		14.93	70.05	0.01	9.42	2.62	36.36	10.06
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*Cuphophyllus CU1*	CHEG	13.48	66.85	0.29	11.34	8.22	5.01	26.82
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*Cuphophyllus CU4*	CHEG	9.72	25.47	0.01	8.03	18.56	2.02	12.08
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*Hygrocybe PS1*	CHEG	5.40	23.29	0.12	3.66	9.74	5.61	2.86
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	*Clavulinopsis CPCO*	CHEG	3.32	12.51	0.00	1.30	5.25	2.11	3.92
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	*Lepista*	Saprobe	3.24	26.55	0.00	6.75	2.76	0.17	2.70
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	*Clavulinopsis CPLA*	CHEG	2.96	9.72	0.21	4.19	1.50	5.17	0.53
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	*Clavaria CVFR*	CHEG	2.41	16.77	0.00	2.46	6.74	0.08	1.17
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*Hygrocybe HY2*	CHEG	2.17	6.46	0.07	2.46	3.67	2.07	1.58
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Sorocybe*	DSE	2.15	4.13	0.81	2.87	2.49	2.21	1.42
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*Gliophorus*	CHEG	1.77	5.97	0.00	4.76	1.55	2.22	0.41
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*Hygrocybe HY5*	CHEG	2.29	22.03	0.00	0.19	0.68	7.08	0.03
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	*Clavaria CVZO*	CHEG	1.17	5.73	0.00	3.92	1.44	2.25	0.27
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	*Clavulinopsis CPFU*	CHEG	1.50	9.83	0.04	3.16	0.20	2.35	0.12
Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	*Solicoccozyma*	Yeast	1.45	3.22	0.52	1.62	1.94	0.92	1.31
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	*Mortierella*	Saprobe	1.37	2.65	0.11	1.59	1.74	0.49	1.58
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	*Hygrocybe PS4*	CHEG	1.43	5.27	0.00	0.34	0.80	1.93	1.88
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus 3*	AMF	1.21	2.45	0.35	0.93	1.68	0.77	1.38
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	*Trechispora*	Saprobe	1.07	4.01	0.19	1.57	0.58	1.80	0.38
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	*Clavaria CVAC*	CHEG	0.86	1.73	0.27	1.06	1.40	0.57	0.78
Fungi Total (number of sequences)						32676	47345	21380	32915	29608	38568	30206
Non Fungi Total						1340	2373	80	1278	1341	1271	1405
Inverse Simpson Index (Taxa)						9.45	26.84	2.02	13.17	10.72	5.87	8.60
Shannon Index (Taxa)						3.11	4.13	1.68	3.35	3.38	2.70	3.05
Taxa Count						304	426	148	314	304	290	302
Inverse Simpson Index (OTU)						10.87	33.23	2.02	15.36	11.96	6.53	10.13
OTU Count						391	517	193	402	396	372	390
Shannon Index (OTU)						3.41	4.52	1.79	3.66	3.69	2.96	3.37

Treatment effects were more evident when abundances of individual genera and ecological groupings were analysed. Treatments were found to have significant effects on fungal community structure (PERMANOVA, p = 0.002, Figure 5.2), and on broad ecological function (PERMANOVA, p = 0.002). Further analysis revealed that nutrient effects were found to significantly alter overall fungal community structure (PERMANOVA, p = 0.01), but stocking effects were not.

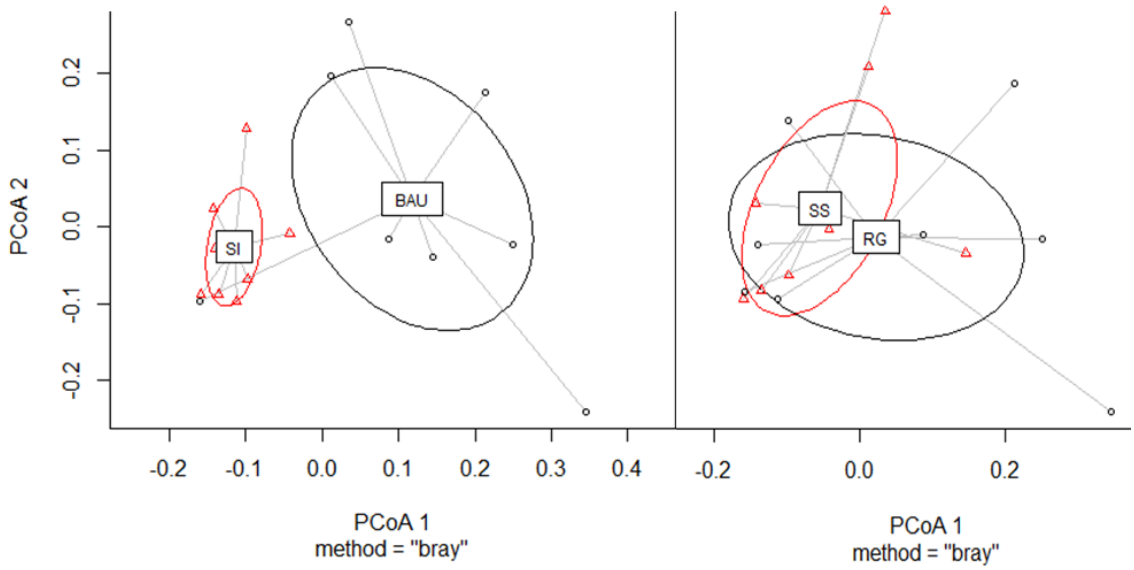

Figure 5.2: Principal Coordinates Analysis of fungal community structure across all samples showing the effect of nutrient treatments (left) and grazing regime (right). Ellipses represent a 95 confidence interval. Clear separation between the nutrient treatments was apparent, but not for grazing regime effects (4 samples in each sampling area, bulked to one homogenised sample per sampling area prior to sequencing, 16 bulked samples in total)

Broad ecological groupings were assigned to taxa where possible (Figure 5.3). The most abundant grouping were CHEG fungi (Clavariaceae, Hygrophoraceae, Entolomataceae and Geoglossaceae). CHEG fungi constituted a mean relative abundance of 55% across all treatments. No significant differences were observed between treatments on overall CHEG abundance, but changes in fungal population structure became more apparent when individual taxa were examined. Nutrient-amended treatments were associated with significantly higher relative abundance of *Cuphophyllus* CU4 (Two-way ANOVA, p = 0.04), but the relative abundance of *Clavulinopsis* CPLA was significantly reduced (Two-way ANOVA, p = 0.02). No combined effects were observed between treatments.

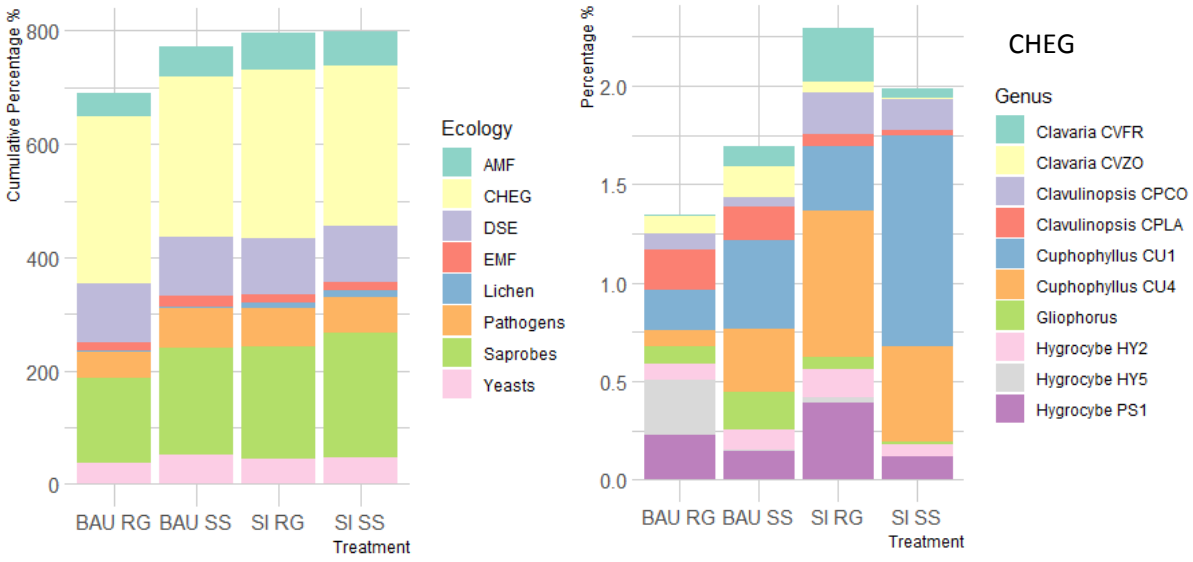

Figure 5.3: Stacked barcharts showing (left) log-transformed abundance of the eight broad ecological groupings assigned to taxa across the four treatments and (right) the top ten most commonly occurring CHEG fungi occurring across all treatments (4 samples in each sampling area, bulked to one homogenised sample per sampling area prior to sequencing, 16 bulked samples in total)

5.4.2. Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi comprised a mean relative abundance of 1.61 % of all sequences analysed across all samples. Nutrient effects were found to have a significant effect on arbuscular mycorrhizal fungi abundance (Two-way ANOVA, p = 0.02, Figure 5.4), but choice of grazing regime did not, and no combined effects were detected. Increased relative abundance of arbuscular mycorrhizal fungi was observed in rotationally grazed treatments after nutrient amendments (TukeyHSD, P = 0.05). Differences between treatment effects were more observable on individual AMF taxa. Rotational grazing significantly increased the relative abundance of *Acaulospora* (Two-way ANOVA, p = 0.02). *Ambispora* relative abundance was significantly reduced by nutrient additions (Two-way ANOVA, p = 0.004), and a combined effect of stocking and nutrients also contributed to reduced abundance (Two-way ANOVA, p = 0.03). In contrast, *Claroideoglomus* and *Paraglomus* relative abundances were increased by nutrient additions (Two-way ANOVA, p = 0.01 and 0.03, respectively). While no overall effects of nutrient amendments or grazing regime affected overall DSE structure, some significant effects were detectable on the relative abundances of individual taxa (Figure 5.4). The most abundant DSE genus was *Sorocybe*, and significant combined effects were detected on relative abundance (Two-way ANOVA, p = 0.05). Rotational grazing increased *Sorocybe* abundance under nutrient amendment treatments, but reduced relative abundance in non-amended plots. Nutrient amendments significantly increased *Lachnum* relative abundance (Two-way ANOVA, p = 0.01), but was reduced under rotational grazing (Two-way ANOVA, p

= 0.03), and no combined effects were detected. *Veronea* abundance was also increased under nutrient-amended treatments (Two-way ANOVA, p = 0.04), as was the relative abundance of *Tetracladium* (Two-way ANOVA, p = 0.02). Rotational grazing was associated with a significant reduction in *Graddonia* relative abundance (Two-way ANOVA, p = 0.04).

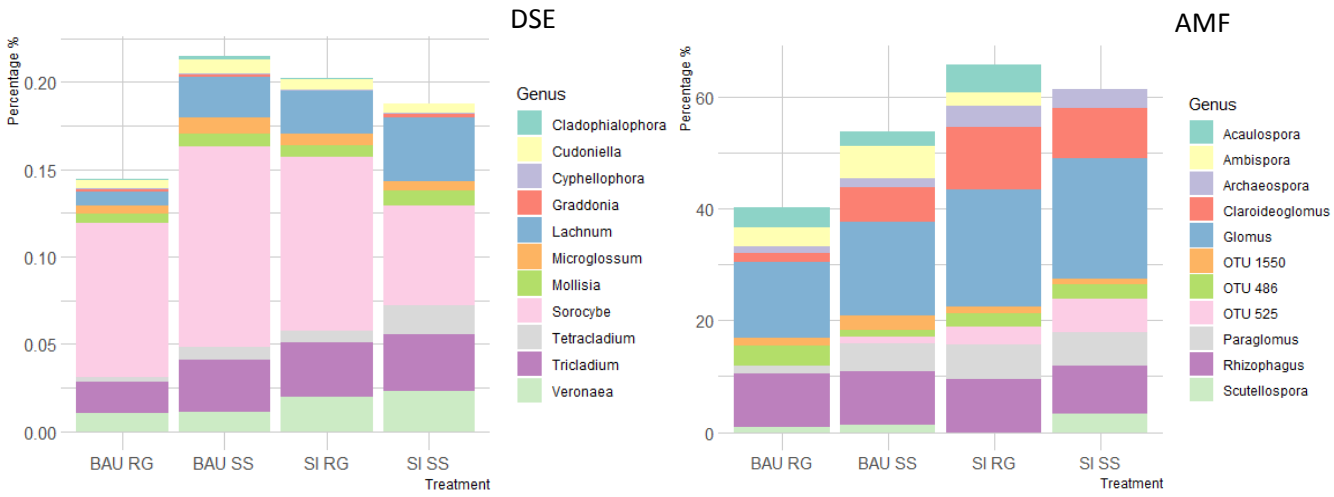

Figure 5.4: Stacked barcharts showing mean abundance of the ten most abundant dark septate endophyte genera (left) and the log-transformed mean abundance of the top ten most commonly occurring arbuscular mycorrhizal fungi (right) observed across all treatments. All AMF OTUs unassigned to genus level were Glomeromycetes (4 samples in each sampling area, bulked to one homogenised sample per sampling area prior to sequencing, 16 bulked samples in total)

5.4.3. Pathogenic fungi

Pathogenic fungi were analysed for variance. The most abundant pathogens across all treatments were smuts and plant pathogens. The relative abundance of smuts (pathogens of grasses and sedges) was significantly reduced by rotational grazing in treatments with no nutrient inputs (ANOVA, $p = 0.05$). Effects were more evident when individual taxa were examined for variance under different treatments. Significant increases in the relative abundance of the genus *Cochliobolus* were associated with nutrient additions (Two-way ANOVA, $p = 0.001$), as was relative abundance of the smut genus *Entyloma* (Two-way ANOVA, $p = 0.01$). The relative abundance of the sedge-pathogen genus *Kriegeria* was significantly increased under nutrient-amended treatments and by set-stocked grazing (Two-way ANOVA, $p = 0.02$ and 0.02 for both). Both effects were shown to act in combination (Two-way ANOVA, $p = 0.001$). A combined effect of set stocking and nutrient amendments was shown to significantly increase relative abundance of the genus *Physoderma* (Two-way ANOVA, $p = 0.001$).

5.4.4. Saprobic fungi

Treatment was found to have significant effects on saprobic fungal community structure (PERMANOVA, $p = 0.002$, Figure 5.5). The general role of saprobic fungi in nutrient cycling was of interest in this study, and saprobic fungi were found to be most abundant overall in non-amended treatments that were set-stocked, but decreased under rotational grazing (not significant). Nutrient-amended treatments showed no difference in overall abundance, regardless of grazing regime. The abundance of the genus *Mortierella* was significantly increased under nutrient-amended treatments (Two-way ANOVA, $p = 0.04$), which acted in combination with rotational grazing (Two-way ANOVA, $p = 0.05$).

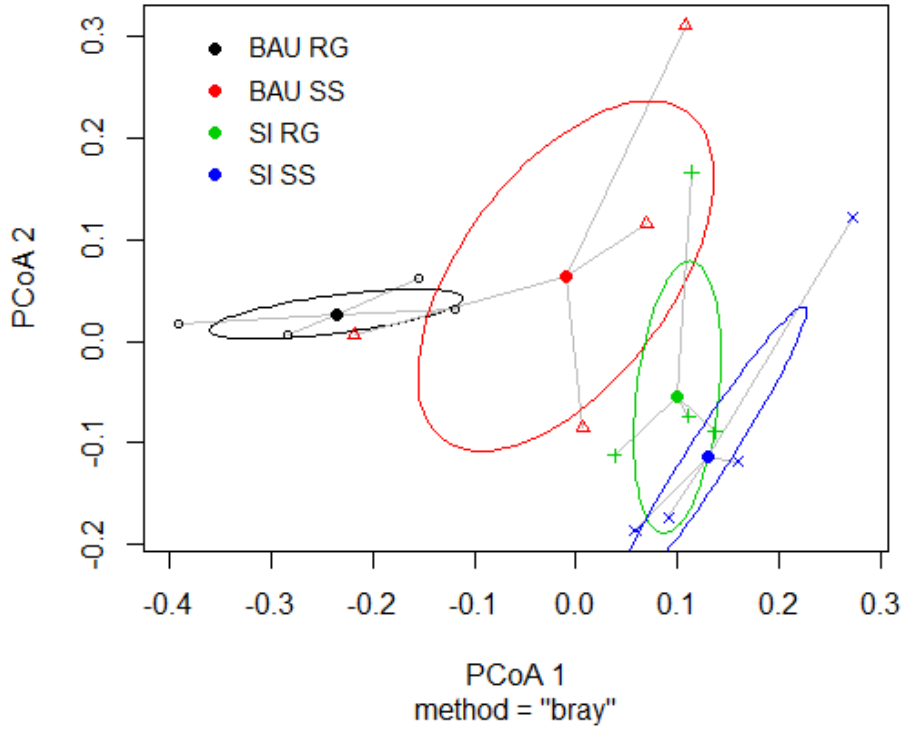

Figure 5.5: Principal Coordinates Analysis of saprobic fungal community structure across all samples showing all treatments. Ellipses represent a 95 confidence interval, some separation between the treatments was apparent (4 samples in each sampling area, bulked to one homogenised sample per sampling area prior to sequencing, 16 bulked samples in total)

5.4.5. Soil yeasts

No overall effects of grazing or nutrient regimes were found to affect overall fungal yeast relative abundance, but effects became more apparent at the genus level. A significant combined effect of stocking regime and nutrient application was observed on *Solicoccozyma* abundance (Two-way ANOVA, $p = 0.03$), with abundance decreasing in non-nutrient-amended plots when rotational grazing was introduced, but with abundance increasing in nutrient-amended plots when rotational grazing was introduced. *Sporobolomyces* relative abundance decreased under rotational grazing (Two-way ANOVA, $p = 0.03$), but was increased with nutrient amendments (Two-way ANOVA, $p = 0.02$). These effects did not act in combination.

5.4.6: Higher taxonomic groupings

Some variance between fungal orders was observed across treatments. Most noteworthy were overall changes to the order Pleosporales, which is the largest order in the fungal class Dothideomycetes. Nutrient effects were particularly obvious, with nutrient-amended treatments associated with significantly higher Pleosporales relative abundance (Two-way ANOVA, $p = 0.007$), and set-stocked treatments being associated with significantly greater Pleosporales relative abundance than rotationally grazed plots (Two-way ANOVA, $p = 0.03$).

5.5. Discussion

In this study, a sustainable intensification approach that consisted of lime, nitrogen, phosphorus and potassium applications was found to significantly change the fungal community structure of the soil. Of particular interest in this study were number of genera associated with plant growth promotion, some of which are described in earlier chapters. A recurring theme in reviews regarding biostimulants and their efficacy is a failure of laboratory-based trials examining effects of specific plant growth promoting microbes to replicate field conditions (Du Jardin, 2015; Storer *et al*; 2016), and crucially, fail to account for a number of key factors that are being recognised as increasingly important in terms of understanding biostimulant efficacy. These factors include the inability of pot trials to replicate the abiotic properties of field soil, such as the climate, the physical properties and rapid nutrient depletion of the soil. More importantly, however, is the behaviour of resident soil microbial communities, and therefore priming effects, priority effects and niche overlap (Santaniello *et al*., 2012; Hart *et al*., 2018; Adamczyk *et al*., 2019).

Therefore, this study is concerned with interpreting the behaviour of microorganisms associated with plant growth promotion under representative sustainable intensification approaches, which in all likelihood will be part of integrated management approaches to farming in the long-term (Wynne-Jones *et al*, 2020; Garnett *et al*., 2013). Biostimulants are increasingly being advocated as a component of a wider, more integrated approach to soil and crop health than simply a means of introducing supposedly beneficial microbes into any soil and any crop (Awasthi, 2019), which may well offer no benefits to crop yield, waste resources and interfere negatively with soil ecology. Going forward, it is essential to integrate an understanding of representative agricultural soil ecology with any kind of strategy for effectively using biostimulants. If a given species of plant growth promoting microorganism is to be used in a biostimulant formulation, it is not enough simply to demonstrate that it has plant growth promoting effects. It must also be shown that it can survive in field environments (Hart *et al*., 2018; Kaminsky *et al*., 2019, and it must be understood how it will be effected by the likely management of agricultural systems that will be likely in the future (in this case, two representative sustainable intensification approaches, with and without rotational grazing).

Of these potentially important genera introduced in previous chapters, the genus *Mortierella* are well-represented in the soils examined in this study (a mean of 1.37 % abundance across treatments), and corresponds with similar studies of similar soils (Oates *et al*., 2012; Clasen *et al*., 2020; Detheridge *et al*., 2016). There is now evidence that *Mortierella* is positively correlated with soil nitrogen levels (Detheridge *et al*., 2016), which confirms the value of the genus as a key indicator associated with nutrient levels. The present study confirms these

findings, with abundance of *Mortierella* increasing with nutrient treatments and with rotational grazing, which is likely to be due to the concentration manures associated with intensified stocking density. It has been proposed elsewhere that *Mortierella* can mobilise inorganic legacy P by secreting organic acids (Zhang *et al*., 2014), and can increase crop P uptake in pot trials by acting in combination with arbuscular mycorrhizal fungi (Osorio and Habte, 2013). In Chapter 4, evidence was presented that linked P-amended treatments to increases in the genus *Mortierella* which were observed as the most abundant genus observed in the highest-yielding plants. However, there were no obvious associations found between *Mortierella* relative abundance and improved crop P uptake, although the genus was found to be more abundant in soils which had higher plant available P. In this study, *Mortierella* OTUs were most abundant under the rotationally grazed sustainable intensification approach, least abundant under the rotationally grazed business as usual approach and approximately equivalent under all set stocked treatments. This may be due to the fact that 'business as usual' treatments were P limited (Sommerfeld *et al*., 2013), but may also be influenced by the concentrated nutrient inputs from livestock from rotational grazing treatments (Wang *et al*., 2018; Oates *et al*., 2012). If in the future it is demonstrated that *Mortierella* can actually be linked plant growth promotion via increased P nutrition, then an optimistic view could be that the species could be introduced as part of a biostimulant treatment in soils where it is not appropriate to apply inorganic P fertilisers. Similarly, stocking regime and nutrient application was shown to affect *Solicoccozyma* abundance, which decreased in abundance in plots with no nutrient amendments when rotational grazing was introduced, but abundance increased in nutrient-amended plots under rotational grazing. This yeast genus, formerly part of *Cryptococcus*, has also been identified as affording benefits for crop growth (Detheridge *et al*., 2016). The same caveats apply in this case too, with data on the longer term persistence of the species required before forming conclusions as to whether or not any efforts should be made to increase abundance (Hart *et al*., 2018).

Arbuscular mycorrhizal fungi are species known to form symbioses with plant and bryophyte root tissues. The diversity and species composition of AMF are widely recognised to have a crucial impact on the stability and function of grassland ecosystems (Sommerfeld *et al*., 2013). In this study, the sustainable intensification approaches were associated with a significant increase in AMF OTUs. The findings suggest that rotational grazing treatments under business as usual treatments result in soil registering fewer OTUs of AMF, which is unsurprising and is comparable with findings of similar studies (Oates *et al*., 2012). However, rotational grazing in combination with lime and fertiliser treatments increased the presence AMF OTUs, which may or may not be linked to increased development of beneficial symbioses with crops. It is likely that this change resulted from increased soil pH obtained through the lime amendments and

the P availability increases in the sustainable intensification treatments (Yang *et al*., 2015; Sommerfeld *et al*., 2013), although the broader effects of sustainable intensification approaches on AMF behaviour remain poorly understood and will require further investigation. This could introduce a possible caveat for any studies which report increased AMF in field soils as a result of biostimulant treatments, as sustainable intensification approaches could be linked with increased AMF regardless. It is difficult to know whether biostimulants could be more effective at introducing AMF to field soils than the sustainable intensification approaches investigated in this study, but in any case it may not even be desirable to increase AMF in all soils for all crops, which may not benefit from symbiosis if the fungi are unable to offer plants any additional nutrients, and the metabolic cost may outweigh the benefit (O'Callaghan, 2016; Parnell *et al*., 2015; Kaminsky *et al*., 2019).

Existing research suggests that community composition and abundance of a series of several specific fungal taxa play important roles in determining soil productivity (Wang *et al*., 2018). A principal feature of the present study was that in combination, lime (and nutrient) inputs and rotational grazing result in relatively lower overall fungal OTU reads than untreated soils. With fertiliser inputs used in sustainable intensification systems, nutrient and lime treatments may attenuate decreases in fungal diversity (Sommerfeld *et al*., 2013), but may also promote the development of fungi which are associated with improved crop growth. In addition to this, these findings suggest that the combined effect of rotational grazing and lime treatments result in a less skewed fungal taxa distribution. In particular, Tricholomataceae OTUs appear to dominate soil cores taken from rotational grazing treatments on set-stocked plots, accounting for around a half of all fungal taxa recorded. In combination with lime treatment however, the distribution of taxa becomes much more even, with *Hygrocybe*, *Clavulinopsis* and *Clavaria* genera represented in greater proportions. Significant increases in the relative abundance of the fungal order Pleosporales were observed, with both set stocking and nutrient amendments being associated with significant increases in relative Pleosporales abundance. Most OTUs assigned to Pleosporales in this study were saprobes, but there was also a number of endophytes and pathogens (most notably *Cochliobolus*). In a study of unfertilised agricultural soils across a number of sites, Wang *et al*. (2018) reported that Pleosporales were especially enriched in crop root communities, with a relative abundance of 40 % in contrast to a bulk soil abundance of 9.52 %. Plant species has been found to be the main determinant of fungal community composition in the root zone (Burns *et al*., 2015). Wang *et al*. (2018) propose that these differences are a reflection of varying abilities of different fungal taxa to colonise root tissues, and that these observations of specific biospheres associated with particular crops are a feature of the more complex interactions between plant hosts, the soil and microbial populations.

The composition of the fungal communities at phylum level was in some important ways unlike results obtained from similar studies employing similar methodologies (Detheridge *et al*., 2016; Hartmann *et al*., 2014; Lentendu *et al*., 2014). In particular, fungi from the phylum *Basidiomycota* dominated the population (80 %, followed by Ascomycota (12 %). Tian *et al*. (2017) compared fungal community structures between varying land types and obtained similar high proportions of *Basidiomycota* to *Ascomycota* in bare land and oak woodland, and suggested that soil pH was the most useful grouping variable for clustering. In general, however, approaches similar to the present study tend to report *Ascomycota* abundance as relatively higher than *Basidiomycota*.

Dicks *et al*. (2018) identify increasing interest in the agricultural sector around developing more reliable methods and knowledge around predicting pest and disease outbreaks. The present study identified significant increases of a number of fungal genera (*Cochliobolus*, *Entyloma*, *Kriegeria* and *Physoderma*) which contain economically important crop pathogens in soils which had received nutrient amendments. In particular, significant increases in relative abundance after nutrient amendments were observed in the genus *Kriegeria*, which contains a number of grass and sedge pathogens. The relative abundance of pathogens in this genus were found to be significantly reduced under rotational grazing, but significantly increased under nutrient-amended treatments. The increases in relative abundance of other such pathogens under nutrient-amended treatments may be suggestive of possible patterns in pathogen abundance to be found in sustainable intensification approaches on different soils and crops. This study indicates that grazing and nutrient amendments may influence some pathogenic genera which may have considerable economic impacts in other agricultural contexts, including *Cochliobolus,* which contains species responsible for leaf spot diseases in apple and maize, *Entyloma* which contain species associated with white smut disease on flowers, and *Physoderma*, which contain some species associated with crown wart and brown spot diseases in corn. These findings will require further investigation to confirm, but suggest opportunities for future research to examine the effects of grazing and nutrient regimes on plant pathogen populations in soil. For grassland systems in particular, future work should look at the impacts of grazing and nutrient regimes on key diseases such as crown rust (caused by *Puccinia coronota*) and leaf spot (caused by *Drechslera siccans*, AFBI, (2014)) in grassland soils. Recently, biostimulants have been advocated as a means of offering benefits to the crops in terms of suppression of pathogens, which can take the form of competing with pathogens for resources in the same metabolic niche, competing with pathogens for access to plant roots, hyperparasitism, and interfering with pathogen metabolism or signalling hormones (Gupta and Vakhlu, 2018; Berendsen *et al*., 2012; Romera *et al*., 2019; Lugtenberg and Kamilova, 2009; Storer *et al*., 2016). In this study, some economically important plant

pathogens showed significantly increased relative abundance under sustainable intensification approaches, which could suggest evidence that there could be value in integrating pathogen-suppressing biostimulants to the approach, however the replication rate in this study was too small for this conclusion to be anything more than indicative for future research. An optimistic view could be that an integrated biostimulant approach could incorporate genera have been demonstrated to pathogen reduction, such as *Trichoderma (*Mukherjee *et al.,* 2013), although all the same caveats apply to this as to the taxa described above, particularly as to whether *Trichoderma* would establish in soils such as these under sustainable intensification approaches. *Trichoderma* OTUs were not detected in any of the soil samples taken, which could suggest that there are reasons why the genus was not present (incompatible niches, competing species).

While the concept of microbial communities positively influencing soil function and crop growth has been well developed and validated in field conditions, understanding of the legacy effects and longer-term implications of the interactions between microbial populations, crops and the soil remains restricted to conceptual model projections (Wubs *et al*., 2019), and therefore a longer-term research programme incorporating considerations of temporal effects is necessitated. Wubs *et al*. (2019) have demonstrated that one-off introductions of microbes can alter plant community composition for periods of 20 years, with above and belowground population composition becoming increasingly correlated. A similar method examining community composition after biota introduction, nutrient amendments and grazing regimes over an extended time periods will be necessary to disclose similar insights into legacy effects of sustainable intensification.

In the present chapter, the objectives were not to measure direct effects of biostimulants, but instead to develop understanding of fungal microbial community interactions with plants and the soil in agricultural contexts, which is vital to improve biostimulant efficacy (Du Jardin, 2015; Owen *et al*., 2015). A feature of the research which will be particularly valuable for developing the biostimulant knowledge base is knowing which taxa associated with plant growth-promotion appear to be influenced by management approach. The results of Chapter 4 suggested that different nutrient amendment treatments play a significant role in determining whether biostimulants are efficacious. In the present study, more evidence has been accumulated regarding the specific interactions between some of these taxa and the soil. Due to the significantly divergent fungal community structure observed in this study under different grazing and nutrient regimes, these results support the view that no biostimulant will deliver consistent benefits across all treatments, soil types and crop varieties, or that a “one size fits all” treatment is unlikely to be developed (Awasthi, 2019; Storer *et al.,* 2016). These findings suggest that a more viable approach for biostimulant treatment success may involve

ascertaining a number of key indicators of soil biology and health before suggesting a formulation of plant growth-promoting species.

As part of the sustainable intensification approach and improve production, fertilisers were necessary to remedy depleted nutrient status and lime was applied to improve low pH. These treatments had considerable effects on the soil fungal community and also entailed a significant environmental and economic cost. However, any inputs in the future will be considerably lower, because they will only be necessary for the maintenance of pH and soil nutrient profiles (Defra, 2019). It will be valuable to compare the structure of fungal communities in the future to assess how soon communities stabilise after the sustainable intensification approaches, and whether increases in AMF and other key interest species in this study actually persist (Wubs *et al*., 2019; Kaminsky *et al*., 2019).

The biggest shortcoming of this study apart from the low replication rate is that biostimulant were not actually applied to the field soil, although this was very much the original intention (logistics, access to the site and interference with other experiments prevented biostimulant application during the period of this study). The samples from each sampling area were pooled to four samples for each treatment, which is too small a replication rate to permit wider conclusions to be drawn, and increased sampling density and frequency should be factored into future experiments. Future research should apply biostimulants as part of a sustainable intensification approach, and vary application rates and application timing. Data on fungal community structure should be taken at multiple time points to measure establishment, priority effects and persistence. Sampling should also be taken further into the future to examine legacy effect and long term persistence. Sampling should be taken outside the experimental site in increasing distance increments to measure whether there has been ingress or spread of genetic material introduced through the biostimulant. Crop phosphorus should also be measured from harvested biomass on treated soils, as well as other measures of mineral nutrition.

5.6. Conclusion

Sustainable intensification is likely to be the way an increasing amount of farmland is managed in the future. If biostimulants are to be used, they must not only be shown to persist and establish in field environments, they must be able to function within these sustainable management regimes. While biostimulants were not actually applied in this study, the work done has shown how certain species that have been purported to be plant growth promotors actually behave under representative sustainable intensification treatments. A longer period of study is required to obtain a better understanding of long-term community structure

differences, and whether differences observed in this study persist in the longer term after amendments are reduced to only maintain the appropriate nutrient status.

5.7. References

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Chapter 6

General Discussion

6.1. General discussion

This thesis sought to fulfil two overarching aims: to examine the efficacy of several commercial biostimulants on crop growth in terms of yield, P uptake and health under controlled laboratory conditions, and to ascertain what extent shifts in fungal populations could explain differences in crop health and growth rate, and framing these in the context of actual microbial communities in field sites.

In recent years, the use of and interest in biostimulants has accelerated, which is a result of a general recognition in agriculture of the need to reduce dependence on non-renewable resources used in fertilising processes, as well as a broader aim of reducing agricultural production's environmental impact (Ruzzi *et al*., 2015; Vassilev, 2015; Rouphael *et al*., 2015; Owen *et al*., 2015). Developments in the understanding of the roles, modes of action and mechanisms of soil microbes, and their interaction with plant crops has driven increases in adoption of biostimulants (Vassilev *et al*., 2015). Uncertainty remains however regarding their functionality, legacy effects and consistency (Storer *et al*., 2016). In Chapter 2, existing evidence was evaluated regarding previously identified microbial modes of action responsible for plant growth, as well as discussion of the different terms used ("biostimulant", "bioinoculant" and "biofertiliser"). Areas where future research is required have been highlighted, which include the need for a greater number of morphometric and physiological variables to be evaluated in biostimulant efficacy trials, and mechanisms of growth-promotion associated with microbial populations present in the soil. Analysis of existing research revealed that biostimulant efficacy is dependent their ability to deliver functionality in conditions whereby crops are subjected to abiotic stress (pH, temperature and ion concentration differences (Lugtenberg and Kamilova, 2009)). It was found that existing products are often found perform poorly in such circumstances (Storer *et al*., 2016). An absence of peer-reviewed research within the literature examining commercial biostimulant efficacy within field environments was found, and existing studies often omit measurements of crucial information that would be needed to clearly understand efficacy. These measurements include the establishment of the biostimulant in the soil (Kaminsky et al., 2019), comparing application rates and methods in the same study, seasonal effects on biostimulant application and efficacy (Hart et al., 2018) and integrating biostimulants with sustainable intensification approaches. There is also a lack of information regarding persistence and spread of biostimulants in the literature, which must be a priority for future studies.

Following the recommendations of du Jardin (2015), where a case was presented that effective research into biostimulant efficacy should utilise high-throughput plant phenotyping platforms to provide more detailed insights into biostimulant modes of action, Chapters 3 and

4 report on two such approaches. A high-throughput plant phenotyping platform was able to discern some plant benefits associated with biostimulant inoculation in *L. perenne* and *A. thaliana* experimental crops, but also reported some possible negative effects associated with biostimulants. Biostimulant treatments were shown to improve several plant growth parameters significantly in some of the treatments, which included area, perimeter and width (Chapter 3) as well as circularity, symmetry and centre distance (Chapter 4). While some of the biostimulants were associated with improved crop P uptake in *L. perenne*, they were associated with reduced crop P uptake in *A. thaliana* despite most of the experimental substrates showing increased P. This could be evidence of metabolic costs to the crop or fertiliser burn (O'Callaghan, 2016; Romera *et al*., 2019). In the case of AMF biostimulants, it was not expected that symbioses would be formed with the non-mycorrhizal host crop, but rhizobia biostimulants also were associated with reduced crop P uptake, which would tend towards suggesting a metabolic cost.

Chapter 3 produced suggestive evidence that mineral effects may have been largely responsible for differences in growth patterns observed in the experimental crop, but Chapter 4 was able to demonstrate that the live fractions of some biostimulants were able to afford growth benefits (in terms of biomass) that were not delivered simply by the carrier substrate. Chapters 3 and 4 highlighted an important role played by the zeolite component of some of the biostimulants. Since Hoaglands solution was used to ensure N was not a limiting nutrient, and the Olsen P test of the soils used in the *Arabidopsis* growing period showed that bioavailable P levels were relatively consistent across treatments, it may be inferred that some of the growth-promoting effects were attributable to the zeolite used in some of the biostimulant carriers, especially as tissue P was not increased by the biostimulant treatments. Chapter 4 was able to suggest to a very limited extent a legacy effect of biostimulant treatments on account of sustained growth and similar growth responses in the experimental crop over the course of three non-destructive cuts taken over a three month period, with similar growth rate differences between treatments observed after each cutting. However, the small pot size and rapid nutrient depletion is not representative of legacy effects in field environments (Clasen et al., 2020; Hartmann *et al*., 2014; Detheridge *et al*., 2016), so other than evidence of some influence of community structure, the longer term legacy effect cannot at all be inferred or even suggested by this data.

As discussed in Chapter 3, the use of zeolite can improve nutrient use efficiency of organic fertilisers due to their high cation exchange capacity (Ahmed *et al*, 2010), which can also reduce nitrification and leaching of N from the soil. In Chapter 3, biostimulants which showed the greatest photosynthetic efficiency comprised of a zeolite carrier, which, along with diammonium phosphate were shown to produce comparable results in treated plants

regardless of whether they were live or sterilised. It was proposed that improved mineral use efficiency explained these findings, but on this information alone it was still not possible to determine the role of the microbial component of the biostimulant treatment. Improved photosynthetic efficiency and water use efficiency was observed in treated plants which also demonstrated greater yields, illustrating the value of reporting measurements such as light response curves in conjunction with morphology and yield data. The work was able to demonstrate that there exists potential to reveal insights into the effect of biostimulants on plant health if similar methods are repeated on a greater variety of crops and analysed alongside soil community data, although the interpretative potential of these measurements is considerably limited in the absence of stress variables (Santaniello et al., 2012), which were not a component of the experimental design in this study, and therefore are only indicative.

Chapters 4 and 5 were able to identify key indicator taxa associated with plant growth promotion, and suggest some possible mechanism. In particular, the roles of the genera *Mortierella* and *Saitozyma* appear to be associated with improved phosphorus use efficiency in plants (Detheridge *et al*., 2016). The study was also able to suggest that some ectomycorrhizal fungi may be able to form beneficial mutualisms or endophytic associations which may improve nutrient use efficiency in crop species, where previously such associations have been largely thought to consist of woody plants. The correlation may not however be an indication of cause and effect, and the abundance of these fungal taxa may be affected by and themselves affect other factors as well as phosphate availability.

In general, the aims of the five experimental chapters were largely satisfied, to the extent that observable differences in crop yields, P uptake, morphometrics and health were observed in the laboratory (Chapters 3 and 4) and explanations for these effect to a certain extent could be explained by examining microbial community structure of treated soils (Chapter 4). Interactive effects of P-fertilisers and biostimuants were successfully measured. The large-scale microbial DNA sequence datasets obtained from the field site described in Chapter 5 provided further insights into the effects of various management practices on soil fungi. The soils of biostimulant treatments and the field trial site were shown to contain diverse populations of fungi (Chapters 4 and 5). In Chapter 4, it was shown that abiotic factors minimally impacted the soil microbe population as laboratory conditions were designed to ascertain specific consequences of introducing biostimulants and P fertilisers to these soils.

The research has been able to suggest some important questions to advise the direction of future research concerning biostimulants. As was demonstrated by the EU's regulations introduced in 2019 which control biostimulant marketing (amended Regulation (EC) No 1107/2009), this changing legislative framework will require accurate information on labelling

species used in formulations and evidence of efficacy. This study sought to identify the fungal taxa present in all the biostimulants included in this research using a DNA metabarcoding process similar to the approaches described in Chapters 4 and 5, but although extraction and PCR have been carried out, COVID-19 restrictions on laboratory access and staff movements has meant that the data has yet to be processed. However, oral communications from the manufacturers and some simple culturing and barcoding techniques were able to ascertain the presence of the species specified in the biostimulants. It has also been reported that batches of biostimulants may vary in consistency and viability (Owen *et al*., 2015), so such DNA based analytics methods for determining biostimulant composition must therefore sample a number of batches for results to be considered robust.

As a consequence of this observed variation in biostimulant batch composition and efficacy, and as reviews of the subject suggest that no biostimulant or species will deliver growth benefits in all circumstances, this study has been able to suggest that a future direction of work could include incorporating species cultured from desirable field environments with healthy microbial populations of similar soil types, allowing bespoke biostimulants to be produced. The experimental chapters suggested that biostimulants are likely to be most efficacious when used in soils where plants are nutrient restricted or (as in the case of Chapter 4) in reducing the impacts of a possible overabundance of phosphorus.

One shared theme across the data chapters is that only leaf P was measured, and therefore it was not possible to know the effects of micronutrient acquisition in the plant tissues that could be associated with biostimulants. In the context of nutrient stripping of soils, the ability of biostimulants to be improve mineral nutrition beyond P is of increasing importance (Fageria and Moreira, 2011; Jones *et al*., 2013). The findings of these studies have demonstrated the need to be cautious when extrapolating results from lab-based trials in controlled environments to application at the field scale. Many commercial biostimulant products are marketed as improving crop yield via increased P mobilisation and uptake (Calvo et al., 2014), which relies on a great deal of evidence relating to modes of action that derive from lab based studies (Du Jardin, 2015). The findings of this study have demonstrated and discussed how different lab-based conditions are in comparison to field environments, mainly in terms of dominant microbial communities. While biostimulant treatments used in these studies increased a number of plant growth parameters considerably, such as shoot growth and biomass (Chapter 3), it was demonstrated that leaf P uptake varied between biostimulants and crops. It was shown that in field environments, resident microbial communities occupied the same niches as microbes associated with biostimulants have been shown to occupy in field trials (Clasen et al., 2020). In particular, grassland communities are dominated by biotrophic CHEG fungi, which were minimally present in the experimental substrate used in

the lab, and it is likely that this group in particular will compete with introduced microbes for resources and affect biostimulant establishment and mutualism formation (Romera et al., 2019). The extent to which this occurs has not been explored, and is a key area for future research.

Another shared theme across the data chapters has been the difficulties encountered with obtaining measurements of soil health and soil biology. The physical and chemical aspects of soil health and functionality are relatively well understood and relatively affordable to test. However, these studies demonstrate the large barriers that exist to a comparable understanding of soil biology (Pellegrino et al., 2015), in terms of cost, method and interpretation. The DNA metabarcoding approach was expensive and time consuming, and due to space restrictions on the chip, replication was reduced and therefore results were less reliable. Although with advances in Next Generation sequencing technology, many of these these methodological constraints are likely to lessen (Lentendu *et al*., 2014), there still remains a great level of complexity in the approach which will continue to be a barrier for some time. The data obtained from the DNA sequencing was targeted at fungi, and consequently was not able to offer comparisons with bacterial diversity, which could have provided a great deal of useful insights and offered a measure of the fungi/bacteria ratio (Martin *et al*., 2012). Whole genome sequencing will be able to address this in time, but at the present it remains a limitation especially if space and budgets are limited.

In any case, DNA metabarcoding only measures OTU abundance, which is not necessarily linked to microbial biomass. While bacterial biomass can be measured by phospholipid fatty acid assays and fungal biomass by ergosterol assays (Grosso *et al*., 2016; Rousk *et al*., 2010), AMF biomass cannot (Ollsen et al., 2003), which limits the interpretive value of such an approach. In any case, these approaches integrated with DNA metabarcoding is both costly and time consuming, and in order to address current knowledge gaps around biostimulant efficacy should analyse samples taken prior to inoculation, during the establishment phase, at different periods in the season, after the harvest and in the years after the treatment to measure long term persistence, as well as from areas around the treatment site to measure the level of spread.

6.2. Recommendations for future research

The research was able to demonstrate the difficulty inherent in quantifying biostimulant efficacy. As was evidenced in this study and in other, commercial biostimulants can vary considerably in performance and viability between batches and deliver substantially different results depending on the soil conditions (Owen *et al*., 2015). In the future, research must be designed to address this issue of produce inconsistency, and a reliable testing method which

considers field conditions, abiotic variables and seasonal effects must be developed. Ideally, this will test the viability of biostimulants across a range of sites, crop and soil types, and over longer periods of time post inoculation.

Laboratory experiments have also shown a considerable tendency to only measure a small number of indicators of crop growth, chiefly being yield (du Jardin, 2015). The range of approaches utilised in the present study suggest that more useful laboratory methods for elucidating biostimulant performance will consider a much larger number of factors than just yield, but will also examine morphometrics, and other plant health indicators, light response curves being just one possible such approach. Leaf P reclamation calculations will also constitute useful data when the robustness of such approaches is evaluated.

As evidenced in this study (Chapters 3 and 4), the use of inert carrier media such as zeolite has significant effects on crops, microbial populations and the soil. Future research should evaluate the impact of the use of such inert carrier media substrates on soil microbial populations and quantify their interactions with the soil and with crops. Possible mutualistic effects between these carriers and biostimulants has been suggested by the present study, and this requires further investigation. Findings may be able to suggest the development of novel carrier substrates, as well as improve methods for biofixing microbes to the carrier to deliver more consistent results between batches.

A principal finding of the study was the possibility of biostimulant legacy effects, evidenced by sustained plant growth after three separate non-destructive cuts in Chapter 3 and by divergent microbial community populations under different treatments shown in Chapter 4. These findings are suggestive, and further research should sample soils at a number of intervals post-inoculation, ideally in both laboratory and field trials, to determine whether legacy effects are sustained for longer periods. If continued over a number of years, it will allow seasonal fluctuation in fungal populations to be accounted for.

The present study suffered considerably from severe space limitations on the Ion Torrent chip, which forced lower replicate numbers. It was originally intended that the approach described in Chapter 4 would include all ten replicates of the fourteen treatments, and that Chapter 5 would utilise the original 16 replicates of each of the four treatments. In any future trial, a larger number of replicates would be desirable to increase overall confidence and reduce variability. The soils used in Chapter 3 would ideally also have been examined using a metabarcoding approach, but this was not possible due to space constraints and the necessity to prioritise samples. The approach used in Chapter 7 would have greatly benefited from comparing sustainable intensification approaches from different sites, which is now possible to the number of sustainable intensification platform experimental sites.

A number of other factors besides phosphorus must also be investigated, which include the responses of biostimulant treated crops to abiotic stressors and the availability of micronutrients such as zinc, aluminium and iron, and their consequences for crop health and yields. In the present study, the light response curve method presented in Chapter 3 allowed some discussion on resilience to abiotic stresses in treated plants, but this approach could be still more useful if combined with some stress-tolerance experiments, such as simulated drought or temperature stresses. This is now possible given recent advances in high-throughput plant phenomics.

Finally, some scope now exists for useful work to be done examining perception among the agricultural community regarding biostimulants. The increase in recent years of biostimulant research and utilisation, combined with recent legislative developments in the EU suggest that research to gauge perception among farmers, fertiliser manufacturers and crop scientist is now very timely, and may result in more diverse use of biostimulants. At present, their use remains largely confined to the arable sector, but it may become apparent that great scope exists for biostimulants to deliver in grasslands as well.

6.3 Conclusion

Overall, while biostimulants have a potentially important role to play in the future of agriculture, much work is still necessary to answer key questions and address challenges which remain regarding how full potential can be achieved. These include improving consistency, viability and the knowledge base surrounding mechanisms and functionality. The present study has demonstrated the importance of experimental methods which consider multiple key indicators regarding biostimulant efficacy, and mechanisms have been proposed for a number of measured beneficial effects.

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Appendix 1

Changes in soil fungal communities of a *Lolium* grass sward under varying management practices

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7.1. Abstract

This study used Next Generation Sequencing Ion Torrent™ molecular diagnostic methods to assess how nitrogen and phosphorus fertiliser application affect soil fungal taxa diversity in two field trials on brown earth and sandy loam soils, consisting of a *Lolium*-dominated grass sward. It utilised an existing plant-available P gradient in an area of the sandy loam soil, and the brown earth site was used to examine nitrogen fertiliser effects. The investigation focused on the D1 region of the large subunit, which can provide good resolution to the genus level. Soil samples were taken from the experimental sites before and after inorganic fertiliser applications (100 kg ha^{-1} N on the N-effects site, and 44 kg ha^{-1} P on the P-effects site). The procedure was able to identify significant shifts in fungal species abundance as a result of fertiliser treatments, soil type and sampling date. *Mortierella* spp.decreased in abundance with added nitrogen. Decreases in *Basidiomycota* abundance was observed over time on the sandy loam site and increases in *Ascomycota* abundance were observed subsequent to nitrogen application, increasing overall fungal diversity. Nitrogen fertilisers were the only treatments which significantly altered fungal diversity; with the impacts on fungal community structure often appearing to be dependent on seasonality. Specifically, the salt-tolerant *Thelebolus* sp. abundance was greatly reduced after nitrogen amendments were made in summer but were significantly increased when amendments were made in autumn. However, *Mortierella* sp. abundance appeared to be greatly reduced when nitrogen fertilisers were applied, regardless of season. Many of these taxa have been identified as important agents in rhizosphere ecology elsewhere, but many functions and keystone taxa remain to be investigated. Our study has demonstrated the role of soil nitrate in determining the abundance of several major ecological groupings, and indicates that further work is necessary to determine the impacts this could have on key microbial-driven soil processes.

Data presented in this chapter was collected by Dr Darren Owen as part of his PhD research, and some of the data presented herein constituted part of his PhD thesis. The authors have written this data up as a paper with intent to submit to Applied Soil Ecology.

7.2. Introduction

It is increasingly important to understand the interactions between plants, microorganisms and soil, so that we can meet the food security challenges of the future in a sustainable way. Given that approximately 70 % of the world's agricultural land is grassland (FAO, 2013), there is a particular need to improve our understanding of these interactions in such systems. Key to this is how agricultural practices affect the soil microbial community, and vice versa (Detheridge *et al*., 2016). The influence of rhizosphere fungi on key soil processes is now widely appreciated, including their role in the cycling of organic matter and nutrients. For example, certain fungal taxa have been speculated to improve plant phosphorus acquisition (Owen *et al*., 2014), and there is increasing interest as to whether such fungi can be exploited commercially. If successful, their utilisation could potentially reduce the need for inorganic phosphorus fertiliser inputs, and the associated economic and environmental cost of use (e.g. from watercourse pollution).

It is widely understood that arbuscular mycorrhizal (AM) symbioses (*Glomeromycota*) benefit grasslands and arable crops; however, the application of more recently developed molecular methods, including Next Generation Sequencing (NGS), has suggested that a wider range of fungi than were previously thought are responsible for beneficial symbioses, affording heightened benefits for plant mineral nutrition (Smith and Read, 2007; Heijden *et al*., 2015). These include the dark septate endophytes, ectomychorrizas, and structures involving intercellular hyphae or hyphal coils (Wiberforce *et al*., 2003; Detheridge *et al*., 2016). The result of a particular fungi/plant host interaction is subject to variation, depending on the combination of plants/fungi involved, and the abiotic conditions in effect, which may dictate the predominance or recalcitrance of particular rhizosphere fungi (Mandyam and Jumpponen, 2014). Inorganic fertilisers serve to administer essential plant mineral nutrition, but they also influence rhizosphere microbial diversity, and in the case of nitrogen, may promote an increase in abundance of pathogenic fungi (Paungfoo-Lonhienne *et al*., 2015). For these reasons, it is important to research the influence of fertilisers on the role and function of keystone fungal taxa, especially in different soils and nutrient statuses, which will aid understanding of productive plant/soil interaction in grassland environments. There have been few studies that examine bulk soil fungal population changes, with the majority focussing on AM fungal communities, interaction with roots, or spores (e.g., Gamalero *et al*., 2009, Calvo-Polanco *et al*., 2016). However, these studies have focussed on tillage and crop rotation (e.g. Detheridge *et al*., 2016), rather than grasslands.

Improvements in DNA-based technologies and methods have granted the ability to examine the role of soil microorganisms in a much greater level of detail than in the past, chiefly by Next Generation Sequencing (NGS). This technology has reduced the costs of bulk soil DNA analysis and provided scope to explore much larger sample sizes. The aims of this study were to use an NGS Ion Torrent platform to investigate the variation of soil fungal assemblage composition in grassland managed under different nutrient input regimes over a number of seasons.

7.3. Aims and objectives

- To examine changes in soil fungal populations and diversity in two soil types over two sampling dates

- To assess the impact of inorganic fertilisers, ammonium nitrate, triple super phosphate and rock phosphate on soil biospheres

- To examine the nature of mechanisms influencing plant-soil-microbe interractions

7.4. Materials and methods

7.4.1. Field site and climate conditions

A field experiment was carried out at Bangor University's research farm, Henfaes Research Centre, Abergwyngregyn, Wales, UK (53°14' N and 4°01' W). The climate regime of this region is temperate-oceanic, with an average temperature of 9.8 °C, and average annual rainfall of 800 mm (Farrell *et al*., 2011). The soil series is a Eutric Cambisol, and chemical characteristics of the upper soil layer (20 cm) before sowing were: pH 5.5, cation exchange capacity 14.65 mEq 100 g^{-1}, 5.6 % total organic matter, 12 mg kg^{-1} inorganic nitrogen, 21 mg kg^{-1} plant-available phosphorus (analysed by The Glenside Group Ltd. (Livingston, UK) and Sciantec Analytical Services Ltd. (Berkshire, UK)).

7.4.2. Site preparation and nutrient input

The experiment was conducted on a two-year ley of a *Lolium perenne*-dominated pasture and managed in accordance with the Defra Fertiliser Manual (RB209) recommendations, which are regarded as best practise (Defra, 2010). Within the site, two experimental plots were established; one on brown earth (BE), and the other on sandy loam (SL) soil.

7.4.3. Inorganic nitrogen fertiliser effects trial (BE)

The brown earth site (24 m x 6 m total size, each treatment plot measuring 2 m^2) was used to examine the effect of inorganic nitrogen fertiliser on fungal communities and received a total of 100 kg N ha^{-1} in the form of 34.4 % ammonium nitrate fertiliser, with P and K maintained as recommended for grassland systems according to RB209 (Defra, 2010). N applications were split, with the first 50 kg ha^{-1} applied in June, in two applications of 25 kg N ha^{-1} applied seven days apart. The first yield cut was taken in July. The second 50 kg N ha^{-1} was applied after the first yield cut. The second yield cut was taken in September. Five soil cores (10 cm depth, from within a 1 m^2 area within each plot), were taken in June before and after the application of 25 kg N ha^{-1}, in July after the second yield cut.

7.4.4. Phosphorus effects trial (SL)

The sandy loam site was used to examine the effect of inorganic P fertiliser and existing soil P on soil fungal community structure. The site was divided into two subplots, SLa (18 m x 4.3m), and SLb (18 m x 13.5 m), to best utilise an existing P gradient in SLa (varying plant-available P levels between 0 – 30 mg l^{-1}, P index of 0 – 3; for an explanation of soil nutrient indices, see RB209 (Defra, 2010)).SLb had a consistent plant-available P level of 8 mg l^{-1} (P index of 0). Target soil indices for grassland contain plant-available P levels at between 16-25 mg l^{-1} (P index 2). Each treatment plot measured 2 m^2. P treatments were applied to SLb and consisted of Highland slag rock phosphate (RP, 15 % PO_4^{3-}), and triple super phosphate (TSP, 46 % PO_4^{3-}), applied at a rate of 44 kg ha^{-1} P (equivalent to 100 kg ha^{-1} P_2O_5 (Defra, 2010)). N and K were maintained as recommended for grassland systems according to RB209 (Defra, 2010). In SLa, two yield cuts were taken (May and August).Five soil cores (10 cm depth, from within a 1 m^2 area within each treatment plot) were taken in August. In SLb, soil cores were taken in April and August (after NK application).

7.4.5. DNA amplification and sequencing

Soil samples were frozen at -80°C, and freeze-dried. Each sample was then ground through a <1 mm sieve to ensure homogeneity. Using a PowerSoil® DNA extraction kit (MoBio Laboratories, Solana, CA, USA), 200 mg of the processed soil was extracted. The fungal DNA was amplified using specific primers (the D1F1 forward primer (YYAGTARCTGCGAGTGAAG) and the NILC2 AF reverse

primer (GAGCTGCATTCCCAAACAA)). The forward primers were linked at the 5' end to an Ion Torrent™ primer, a calibration sequence and an individual barcode sequence (allowing multiplexing). The reverse primers were linked to Ion Torrent™ primers at the 5' end. The PCR procedure involved denaturation at 94°C for five minutes, followed by 30 cycles of 30 s duration at the same temperature. This was followed by annealing at 52°C for 30 s, extension at 72°C for 30 s and a final extension step of 72°C for five minutes. PCR reactions were cleaned using spin columns (NBS Biological, Huntingdon UK: NBS664), and the amplified DNA was quantified using a NanoDrop 1000 Spectrophotometer V3.7 (NanoDrop Technologies, Wilmington, USA). Samples were pooled in equimolar concentrations, and further purified using E-gel (Thermofisher, Paisley, UK), extracting strands in the region of 300 bp length. Quantification was carried out using a Bioanalyser 2100 (Agilent, Santa Clara, USA) with an Agilent High Sensitivity DNA chip (Agilent reference 5067-4626). By targeting the approximately 200 bp D1 variable region of the large sub-unit ribosomal DNA, genus and phylogenetic identification was carried out through the Ribosomal Database Project. A two-step dilution was carried out to adjust the samples to a final concentration of 12 pM. Emulsion PCR was run using Ion Torrent™ One Touch 2 system using Ion PGM™ Template OT2 solutions and supplies kits, following manufacturer instructions, adapted for 12 pM rather than the suggested 26 pM to prevent polycolonal beads. Enrichment was carried out using the Ion OneTouch™ enrichment system, in order to remove beads without attached DNA strands (non-template particles). Sequencing was carried out using an Ion PGMTM Sequencing 200 v2 kit on a 316-V2 (100 bp) chip.

7.4.6. Sequence data processing

Data was downloaded in BAM format, and unpacked to standard format using PICARD software. Data quality was checked and demultiplexed using MOTHUR (Schloss *et al*., 2009). An average quality score of >15 for the whole sequence was used as well as a moving average quality check over 30 bases with an average quality score of >11. Data was split by barcoded primer using MOTHUR. Sequences were checked for chimeric sequences using the UCHIME function of USEARCH (Edgar *et al*., 2011) against a reference database of fungal LSU sequences obtained from the Ribosomal Database Project (RDP) website (Cole *et al*., 2014). Operational taxonomic units (OTUs) were assigned using USEARCH/UPARSE (v7 (Edgar, 2013)). A taxonomy was assigned to each OTU using the RDP Naïve Bayesian Classifier (Wang *et al*., 2007). Where genus could not be assigned by the classifier, an OTU identifier was allocated to that cluster. Data were then rendered

in Excel to show relative abundances and standardized by dividing the number of reads in each taxonomic unit by the total number of fungal reads in each sample, providing relative abundances of the assigned taxa for each quadrat. Non-fungal taxa were reported separately. In addition, the broad ecological function of the fungi identified were assigned to each taxa where known at genus or family level through searches of academic literature (Tedersoo *et al*., 2010; Mandyam and Jumpponen, 2014), following an approach similar to Detheridge *et al*. (2016). Shannon and Simpson values were calculated as follows:

Shannon:

$$\left(H = -\sum_{j}^{s} = 1 P_i ln P_i \right)$$

(*pi* = relative proportion of the *i*th taxa)

Simpson:

$$E_H = \frac{H}{lnS}$$

(EH assumes a value between 0 and 1).

7.4.7. Data analysis

Using the R package (R Studio, 2018) and PAST.exe (version 3.06), visualisations of relative abundance matrices were produced, using non-metric multidimensional scaling (NMDS) ordination to identify patterns in the data, based on the Bray-Curtis similarity index. Phylum percentage data was log-transformed and the data were normality checked (Shapiro-Wilk). Analyses of variance (ANOVA) tests were performed on the ordination axes to determine if data varied on the primary or secondary axis. Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in fungal community data between treatments and with time. Diversity indices (H and EH) of rarefied date were analysed, by calculating proportional OTU composition to remove variability in sequence read numbers between the chips. Results from all tests were considered significant at the $p < 0.05$ confidence level.

7.5. Results

Across the 168 soil samples which were analysed (83 BE and 85 SL), the mean number of sequences obtained after quality control for the brown earth site was 17,400 per sample, the highest being 34,511 and the lowest being 583. The mean number of sequences obtained after quality control for the sandy loam site was 30,370 per sample, the highest being 61,858 and the lowest being 5529. There were 306 named taxa identified, and of these, 83 % were fungal, the remainder being mostly belonging the genus *Cercozoa* (kingdom Rhizaria) which accounted for 17 % of the non-fungal sequences cross all samples. Ascomycota were the most abundant fungi (mean 53 %), followed by Basidiomycota (mean 32 %) and Chytridiomycota (mean 2 %). More than 77 % of fungal sequences were identified to family and more than 67 % identified to genus (Table 7.1).

Table 7.1: Summary statistics for all sequencing runs

Seqs ID to family	77.87 %
Seqs ID to genus	67.88 %
Ascomycota	53.12 %
Basidiomycota	31.53 %
Fungi incertae sedis	7.58 %
Glomeromycota	1.06 %
Fungi Total	23857.29
Non-Fungi Total	4808.591
Inverse Simpson Index	16.22789
Shannon Index (Taxa)	3.455691
Taxa Count	292.9516
OTU Count	357.9677

The most abundant genera were the Ascomycetes *Veronaea* and *Tricladium* (8 % and 6 % of all reads, respectively) and *Mortierella* (4 % of total reads).

7.5.1. Site effects

PERMANOVA analysis of the sequence data showed site to have a significant effect on individual fungal taxa abundance (P = 0.0001). Several fungal phyla differed significantly in abundance between sites. Basidiomycota were more abundant at MG ($p < 0.001$), whereas Chytridomycota and Glomeromycota were more abundant at BE ($p < 0.001$). No significant differences were observed in the diversity of species between the sites. NMDS ordination showed the separation of the fungal community data between each site (Figure 7.1).

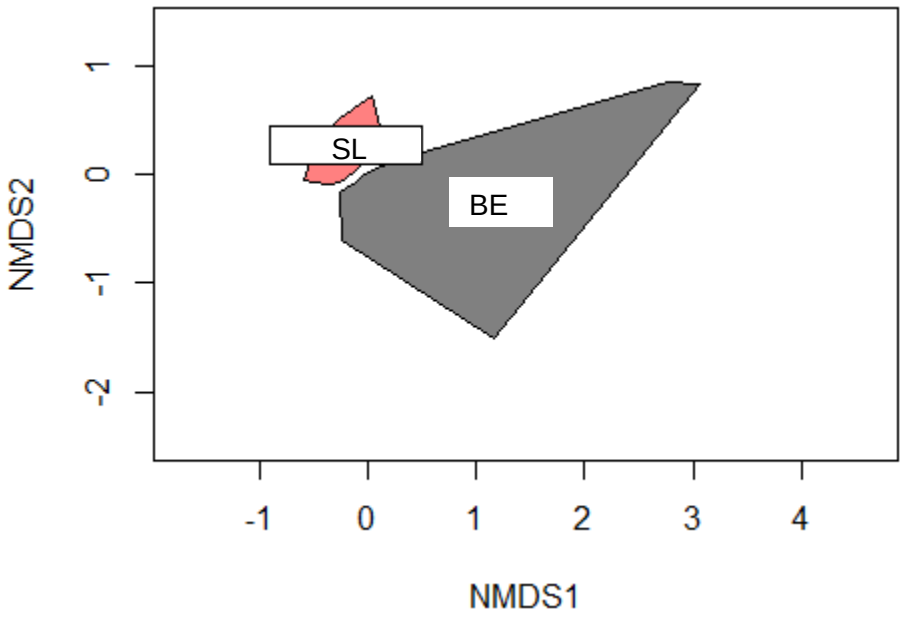

Figure 7.1: NMDS ordination of fungal community data from BE and SL sites. Clear separation was apparent.

7.5.2. Sampling date effects

Sampling date was found to have a significant effect on fungal taxa abundance in both sites (PERMANOVA, for BE p = 0.002 and SL p = 0.019). Sampling date demonstrated significant effects on fungal phyla in low P soil (SL), with Ascomycota (p = 0.04) and Glomeromycota (p = 0.014) significant increasing in abundance in September, and Basidiomycota abundance being

significantly reduced (p = 0.02). NMDS ordination was able to discern clear separation between sampling dates on SL (Figure 7.2).

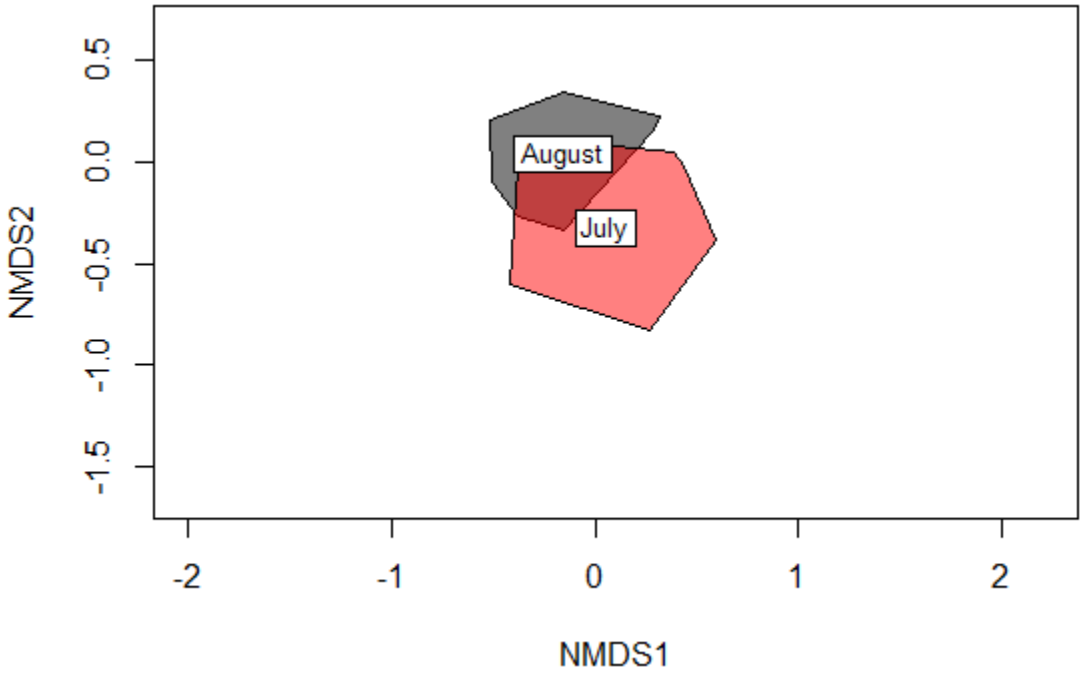

Figure 7.2: NMDS ordination of sandy loam site fungal community data, sampling in July and August 2013. Separation was apparent.

Mortierella remained consistently high in abundance between both sampling dates. *Ascomycota* taxa such as *Thelebolus* were more abundant in July compared to September on brown earth soil, but not significantly so. However, *Pyrenochaeta* and *Didymella* were both significantly more abundant in September than July. The Basidiomycetes *Conocybe* and *Coprinopsis* were nearly twice as abundant in July than September in the low P sandy loam soil ($p < 0.05$). The *Ascomycetes Veronaea* and *Penicillium* were the only taxa to show significantly different abundances between sampling dates in sandy loam low P soil, both with higher abundance in September than July. NMDS ordination illustrated Ascomycota abundance variation between sites but not Basidiomycota abundance variation.

7.5.3. Inorganic nitrogen fertiliser effects

Inorganic nitrogen fertiliser was found to have significant effects on fungal community diversity. Control plots of the brown earth, higher P soil were sampled in June and then again one week later, after the application of 25 kg ha^{-1} ammonium nitrate. Multivariate analysis found these additions to have a significant effect on individual fungal taxa (PERMANOVA $p = 0.002$). *Ascomycota* and *Fungi incertae sedis* were significantly affected by N application, increasing *Ascomycota* abundance ($p = 0.02$; Table 7.2) and reducing *Fungi incertae sedis* abundance ($p = 0.007$). Clear separation was observed in NMDS ordination analysis (Figure 7.4).

The relative abundance of *Mortierella* was significantly reduced after N was applied, falling from 25.2 % to 1.9 %. *Cadophora* and *Gamsiella* abundance increased following N application, from 4.3 % and 0.3 %, to 14.1 % and 10.4 %, respectively. N application increased overall fungal diversity ($p = 0.021$) and equitability ($p = 0.002$). A two-month period from N application resulted in further reduction in *Mortierella* abundance, but also increases in *Didymella*, *Gamsiella* and *Veronaea* ($p < 0.05$).

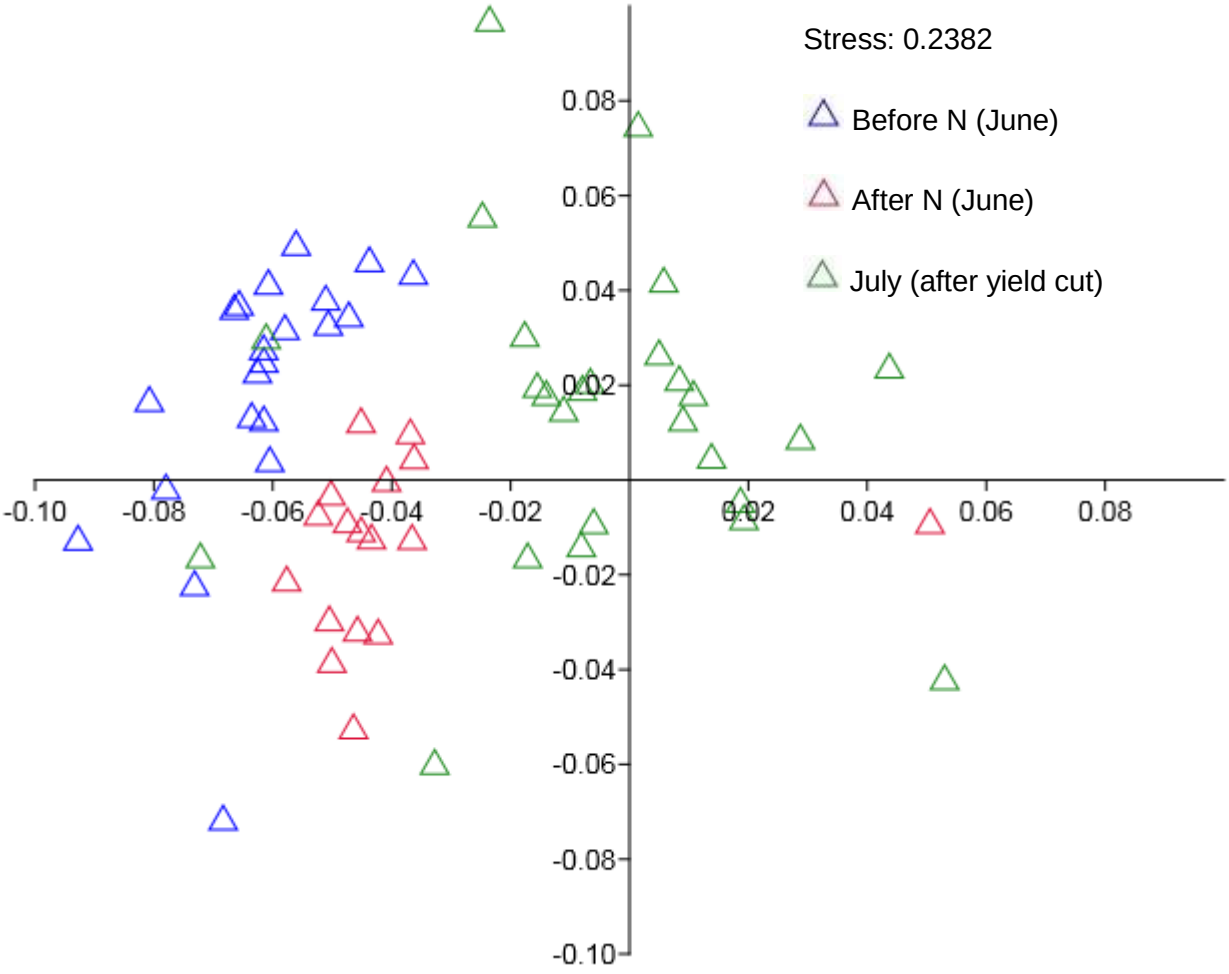

Figure 7.4: NMDS ordination of brown earth site fungal community data, sampling in June and July 2013, before and after N fertiliser addition. Separation was apparent after both nitrogen addition and the yield cut.

Table 7.2. Individual taxa showing significant ($p < 0.05$) changes between treatments: Mean abundance (%) from plots sampled in June before (- N) (*n* = 5) and after (+ N) (*n* = 6) application. Values in parenthesis are ± 1 standard deviation

Genus	Before N	After N
Mortierella	25.2 (± 5.0)	1.9 (± 0.7)
Cadophora	4.3 (± 1.7)	14.1 (± 4.0)
Gamsiella	0.3 (± 0.2)	10.4 (± 5.1)
Pyrenochaeta	0.3 (± 0.1)	1.7 (± 1.3)
Didymella	0.2 (± 0.3)	1.0 (± 0.4)
Betamyces	2.0 (± 0.7)	0.7 (± 0.2)
Cryptococcus	0.7 (± 0.3)	1.7 (± 0.9)

7.5.4. Combined effects of inorganic nitrogen additions over time

There was a significant effect observed of nitrogen application on individual fungal taxa in soil samples between application and sampling two months later (PERMANOVA, $p = 0.0001$). N application was found to be a significant factor for the increase of both fungal diversity ($p = 0.016$) and species evenness ($p = 0.018$). *Basidiomycota* increased in abundance after N application in July, but not in September (Table 9.3). Sample date was shown to be the controlling variable, and that there was a significant interaction between factors (nitrogen application * time, $p = 0.048$). No effect was observed from nitrogen application on fungal phyla abundance. NMDS ordination showed clear separation.

Table 7.3: Mean relative abundance (%) of significant taxa from plots sampled in July / September 2013 before (- N) and after (+ N) application at BE. Values in parenthesis are ± 1 standard deviation. Where there was a significant treatment effect; different superscript letters indicate significantly different means within each sampling date.

	July		**September**	
Genus	**- N** (***n*** **= 5)**	**+ N** (***n*** **= 5)**	**- N** (***n*** **= 6)**	**+ N** (***n*** **= 5)**
Thelebolus	15.0 (± 24.0)	0.8 (± 0.4)	1.68 (± 1.7)	3.25 (± 3.5)
Mortierella	12.2^{a} (± 8.4)	2.8^{b} (± 2.3)	10.2^{a} (± 6.7)	2.5^{b} (± 0.9)
Cadophora	6.1 (± 2.8)	7.4 (± 3.5)	6.6 (± 7.2)	7.2 (± 3.4)
Veronaea	3.9 (± 2.5)	2.8 (± 1.0)	2.9^{a} (± 1.5)	4.8^{b} (± 1.2)
Didymella	1.1^{a} (± 0.8)	4.5^{b} (± 2.5)	7.3^{a} (± 4.9)	1.6^{b} (± 0.5)
Cladosporium	0.5^{a} (± 0.4)	4.8^{b} (± 1.9)	1.1 (± 0.6)	2.3 (± 2.2)
Gamsiella	0.1^{a} (± 0.1)	2.9^{b} (± 1.0)	0.03^{a} (± 0.03)	4.8^{b} (± 3.2)
Waitea	3.1^{a} (± 2.9)	0.3^{b} (± 0.3)	0.07 (± 0.16)	0.01 (± 0.02)

7.5.5. Inorganic P fertiliser effects

No significant effects were observed from the application of either source of phosphate (triple super phosphate and rock phosphate) on fungal taxa abundance. Fungal phyla and diversity variables were not significantly different between P treatments. No separation was observed in NMDS ordination.

7.6. Discussion

The importance of understanding the relationship between agricultural management practices and rhizosphere fungal communities is well recognised (Di Salvo *et al*., 2018, Detheridge *et al*., 2016). Many of the resulting interactions remain poorly understood, and insights into these dynamics could ultimately help improve agricultural management decisions (e.g. nutrient management regimes). This study sought to investigate how fungal abundance and diversity in agricultural soils can differ under different nutrient management regimes on two sites, and the Ion Torrent™ NGS platform used was able to discern clear differences in fungal abundances of these soils.

A diverse fungal community of nearly 400 fungal OTUs was observed across both sites. Fungal assemblages were found to be significantly affected by inorganic nitrogen inputs, soil type, and sampling date. The composition of the fungal communities at phylum level was similar to that reported from similar studies of soil fungi, dominated by *Ascomycota* (52 %), followed by *Basidiomycota* (33 %) and followed by *Glomeromycota* and Zygomycota (Detheridge *et al*., 2016; Lentendu *et al*., 2014; Hartmann *et al*., 2014).

The genera *Mortierella*, *Cadophora* and *Veronaea* dominated the population, which is similar to results of similar studies in similar soils (Detheridge *et al*., 2016). There is now evidence that *Mortierella* is positively correlated with soil nitrogen levels (Detheridge *et al*., 2016), and the results of the brown earth site trial support this. The brown earth soil also had higher abundances of several fungal phyla, *Chytridomycota*, and *Glomeromycota* than sandy loam soil. Brown earth soil was found to be higher in plant-available NO_3^-, NH_4^+ and PO_4^{3-} than sandy loam soil, and soil nutrient status has been shown to be closely associated with fungal community composition (Allison *et al*., 2007, Lauber *et al*., 2008). Recently, George *et al*. (2019) report substantial evidence for intensification of land use having a much greater significance on microbial community structure than soil type.

Nitrogen additions may affect the species composition of soil fungal communities in several ways. The significant reduction in *Mortierella* sp. might suggest a susceptibility to ammonium toxicity (Koops *et al*., 2006); although very few genera of soil microbial communities have demonstrated this (Di Salvo *et al*., 2018). The addition of nitrogen fertiliser to agricultural soils will inevitably stimulate decomposition of organic matter, releasing additional nutrients, in the well-understood positive priming effect (Sheng *et al*., 2013). It is likely therefore that the fungal taxa which responded significantly to the nitrogen inputs were also affected to a certain extent by minerals released by priming (Kuzyakov, 2000).

Nutrient availability can be increased through tillage (e.g., due to mineralisation of immobilised nitrogen) (Sheng *et al*., 2013), and further influence fungal communities. An instance of this would be mycorrhizal features from fungi in the *Glomeromycota* increasing in abundance within plant roots post-tillage (Alguacil *et al*., 2008). In some cases, soils with high levels of plant-available nitrogen can increase the carbon content of root exudates (Dobbelaere *et al*., 2002), which is known to reduce the abundance of nitrogen-fixing rhizobacteria through competition, which can result in additional changes to the whole soil microbial community.

As well as changes in root exudation, plants can alter the soil microbial community through modified root morphology (Nannipieri *et al*., 2008, Bainard *et al*., 2014). Rhizo-deposition involves carbon containing root secretions to provide a substrate for soil microorganisms (Broeckling *et al*., 2008). Plant nutrient availability has a major effect on plant root morphology, and consequently root exudate production. In this way, plants are able to influence fungal community composition (Nannipieri *et al*., 2008). Certain abiotic factors, including temperature, have been demonstrated to affect root morphology, and therefore seasonal variations will control the impact of rhizodeposition on soil fungal communities (Dehaghi and Sanavy 2003).

In the sandy loam soil (SLb), an increased prevalence of *Penicillium* sp. was observed in September. On the brown earth soil site, *Pyrenochaeta* sp. and *Didymella* sp., were more prevalent in September than July, and are known to be saprobes of decaying matter (Câmara *et al*., 2002). These temporal changes observed in fungal taxa fulfilling these ecological functions is in accordance with other studies. Dumbrell *et al*., (2011) observed seasonal shifts in *Glomeromycota* abundance, and in this study, there was increased abundance of the phyla *Glomeromycota* and *Ascomycota* in September, and a reduction in *Basidiomycota*. Resource availability can also influence intraspecific competition and resource allocation in fungal communities (May *et al*., 2007). The sandy loam soil site had low plant-available nutrients (nitrogen and phosphate), and as no application of inorganic fertilisers took place between sampling (July and September), this may have resulted in increased resource competition, since nutrients were depleted further with plant growth.

Many studies of microbial changes in the field focus on fungi within plant roots and rhizosphere (Santos-Gonzalez *et al*., 2007, Dumbrell *et al*., 2011), the dynamic nature of the root rhizosphere impacting on micro-organisms. The more stable.bulk soil may see reduced influence of rhizo-deposits, which have been shown to follow a gradient, influencing less on microorganism diversity (Marilley *et al*., 1998, Kandeler *et al*., 2002, Gomes *et al*., 2003), in which reduced nutrient availability within the bulk soil allow fewer dominant species to proliferate. Within this study, the total number of Glomeromycota taxa sequenced across both sites was twelve, which is quite low compared to similar

studies of MF found within plant roots (Dumbrell *et al*., 2011), potentially reflecting a lower abundance of MF within the bulk soil compared with the plant root rhizosphere.

The D1 region of the large sub-unit targeted within this study, while sufficient to identify Ascomycota and Basidiomycota species (Cole *et al*., 2014) has been criticised for having limited ability to adequately discern Glomeromycota species due to the read length of ~300 base pairs (Stockinger *et al*., 2010), and such a concern is echoed by other studies using the same NGS platform (Brown *et al*., 2013, Kemler *et al*., 2013). The release of 400 base pair chips may help to rectify this and permit greater resolution of soil fungi of more taxonomic groupings, coupled with primers targeting *Glomeromycota* specifically (Kruger *et al*., 2009). Fungal community composition has been shown to be a sensitive indicator of abiotic change (Kaisermann *et al*., 2015).

There were no significant differences observed between sampling times at either site for diversity and equitability measures, suggesting that fungal communities within the bulk soil are affected less by rhizo-deposition seasonality. It could be suggested that the application of inorganic fertilisers would exert effects on rhizospheric and bulk fungal communities. There are mixed reports on the effects of fertilisation on microbial diversity, with both increases and decreases reported (Girvan *et al*., 2004, Allison *et al*., 2007, Alguacil *et al*., 2008, Dumbrell *et al*., 2010, Lin *et al*., 2012, Paungfoo-Lonhienne *et al*., 2015). This study examined the application of a nitrogen fertiliser (NH_4NO_3) and two phosphate fertilisers (TSP and RP) on soil fungal communities. Of these, ammonium nitrate was the only one to have a significant effect on fungal abundance, with both diversity and equitability indices increasing after N application. *Mortierella* sp. (phylum *Fungi incerate sedis*; order *Mortierellales*) was found to be significantly reduced with the application of nitrogen fertiliser. Other studies have shown similar reductions in *Mortierella* sp. with NH_4NO_3 application (Arnebrant *et al*., 1990), the authors attributing the differences to changes in pH. Several studies have shown how pH can be the main driver of microbial community change (Baath *et al*., 1984, Baath and Arnebrant 1993, Rousk *et al*., 2010). The converging convex hulls seen on the NMDS plot of fungal community data (Figure 9.1.) would suggest a response to inorganic N (Girvan *et al*., 2004). Abundance of Basidiomycota was reduced in the September N addition plots, statistical analysis suggesting the decrease was due more to seasonality than fertiliser effects. Prolonged applications of inorganic fertiliser, however, has been shown to have a significant effect on soil fungal communities (Arnebrant, *et al*., 1990). Phosphate effects appeared to have less of an impact on fungal assemblages within this study. N limitation is suggested as a potential cause but also the field conditions during the trial, in which prolonged periods of dry weather followed by heavy downpours of rain shortly after NH_4NO_3 and PO_4 applications may have reduced the effectiveness of the applied fertilisers.

Soil phosphate level was found to have very little effect on the relative abundance of fungi within the soil. The P index is a measure of the plant-available P fraction, and not total P (which did not differ largely between plots); and as a consequence, fungal community structure may not have been P limited in the lower range, as may implied by the presence of the P gradient. Fungal community structure also appeared to be largely unaffected by the variable plant-available P fraction. Several reports have shown how non-native MF species may not be acclimatised to various edaphic factors (Lambert *et al*., 1980, Enkhtuya *et al*., 2000). However, the phosphate level accounted for only 8 % of the variability, and other factors contributed to the reduction in abundance, which would require further study. NGS technology could also be used in parallel with other microbial analysis such as ergosterol assay, which can be used to quantify fungal biomass. Ergosterol is an important sterol in the membrane of most fungi and can be used as an estimate of living fungal biomass, as the membrane is quickly lysed and its components decompose upon hyphal death (Newell, 2001).

7.7. Conclusion

Soil microbial communities affect crop viability through a variety of increasingly appreciated mechanisms. This study explores the impact of a widely implemented nutrient regime on soil fungal communities in two common soils managed for grassland production. The results suggest how sensitive soil microbial communities are to shifts in nutrient profiles as a result of management interventions and illustrate the presence of keystone taxa in these soils which are highly responsive to changes in soil nutrient status. Some features of interest regarding these taxa are identified elsewhere, but many of their properties and roles in soil ecology remain to be explored.

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Appendix 2

Preliminary observations of *Arabidopsis thaliana* seedling development seven days after biostimulant application

8.1. Materials and methods

Two week old seedlings (Col-0) were pricked out into premade weighed pots. Compost was ultralow nutrient peat compost (Bulrush) + 30% silver sand (details in Chapter 3). Rock phosphate (application rate unknown) was added to half of the pots. Three commercial biostimulants were mixed through the compost. Sterilised bioinoculant samples were included together with no inoculant controls. This gave 6 total biostimulant treatments, 14 overall treatments with + and –P controls, 10 reps of each, and 140 total samples.

Plants were loaded on the PSI system (with LED illumination 14 hour daylength, PAR 600 µM m^{-2} sec^{-1}) imaged and watered daily. Plants very quickly started to show various colorations. These were scored 7 days after transfer to the different treatments. Numerical values were attributed to different colorations to allow graphical description of the treatments (Table 10.1 and Figure 10.1).

Table 8.1: Values attributed to *Arabidopsis* colorations

Score	Description	Example
3	Green, healthy plant	
2	Overall purple rosette	
1	Bleached white leaves	

8.2. Results

Mean seedling scores are summarised in Table 8.1 across all treatments. Across all biostimulant treatments, plants growing in non-P amended soils had higher mean seedling scores.

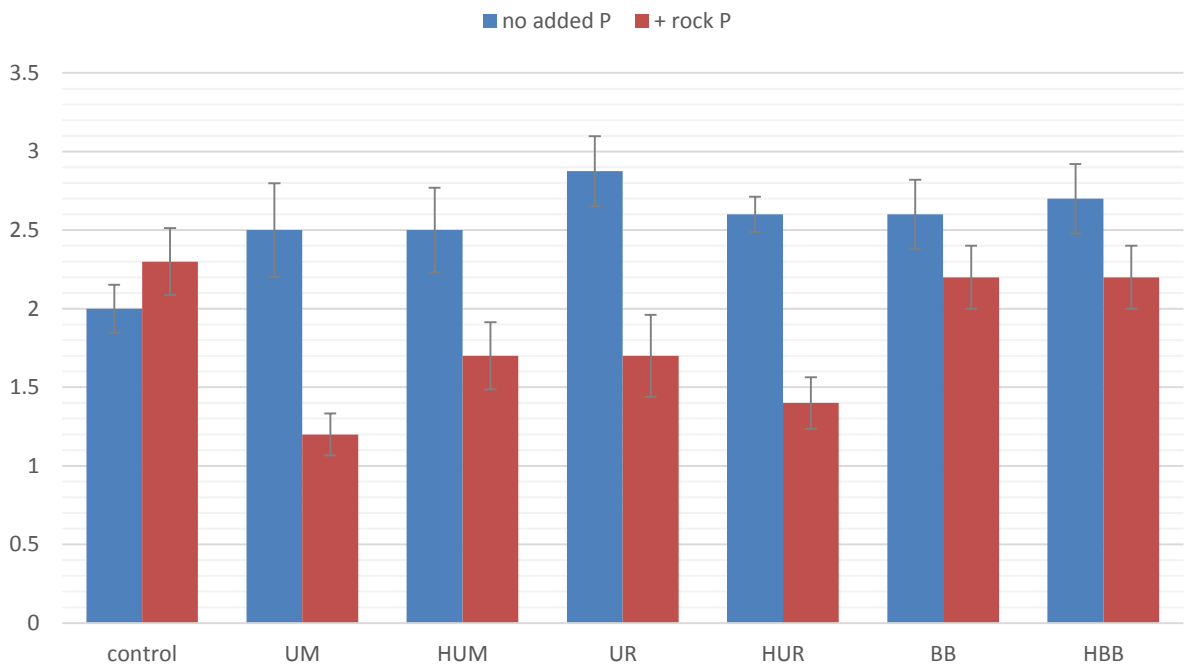

Figure 8.1. Mean colour seedling score (n=10) +/-SE

Appendix 3

A sterol assay method for charactering soil fungal biomass from upland grasslands under different management regimes

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9.1 Abstract

The sterol composition of soils from upland grasslands subject to four different grazing and nutrient management regimes was assessed using a novel high-performance liquid chromatography method. The cholesterol, ergosterol, stigmasterol and sitosterol contents of the soils were measured to indicate how soil microbial community structure is affected by management of nutrients and grazing by livestock. Cholesterol and sitosterol were found to be the most abundant sterols isolated from the soils, and no significant differences in overall sterol composition were detected between treatments. However, a significant combined effect of rotational grazing and the application of lime, P and K were found to increase sitosterol volumes in the tested soils. Sitosterols are found widely across the plant kingdom, and changes in levels observed in soils may reflect changes in vegetation composition, root proliferation and crop stress response. Further research is necessary across a wider selection of sites and samples to determine whether fungal biomass varies correspondingly.

9.2. Introduction

Sustainable intensification of upland grassland has been advocated as a process which improves productivity while simultaneously delivering environmental benefits (Dicks *et al*., 2018). Briefly, the principle of sustainable intensification in the context of ruminant systems is that productivity can be enhanced through optimal management of soils, nutrients, and grassland. In order to deliver these objectives, a thorough understanding of the dynamics between crops, the soil and microbial communities is essential (Detheridge *et al*., 2016). As such, understanding the ways in which sustainable intensification approaches effect these communities is of great importance.

Sterols in the soil are one such key indicator of both microbial community structure and vegetation changes associated with management. While ergosterol has been previously regarded as the sole sterol associated with indicating fungal biomass, it is now known that ergosterol is not present in all fungi, but are readily observed in the most commonly occurring fungal phyla, Ascomycota and Basidiomycota (Weete *et al*., 2010). As such, a thorough approach to quantifying fungal biomass will examine a number of sterols and present the results alongside community data, where OTU abundance and taxa count will help interpret the data. In this study, the levels of four sterols; cholesterol, ergosterol, stigmasterol and sitosterol (Table 9.1) were examined in soil samples taken from a sustainable intensification experimental site, which investigated nutrient and grazing regime approaches (Chapter 7).

Table 9.1: Sterols associated with plant and fungal cell structures (Weete *et al*., 2010)

Sterol	Formula	Plant %	Associated fungal taxa
Cholesterol	$C_{27}H_{46}O$	0—4 %	Taphrinomycotina, Chytridiales, Blastocladiomycota, Entomophthoromycotina
Ergosterol	$C_{28}H_{44}O$	0 %	Most Dikarya, Mucormycotina, Kickxellomycotina, Zoopagomycotina
Stigmasterol	$C_{29}H_{48}O$	0.6—18 %	None known
β-sistosterol	$C_{29}H_{50}O$	45—90 %	None known

9.3 Materials and methods

Treatments were described in detail in Chapter 5, and consisted of i) **BAU SS**: set-stocked, no nutrient inputs, ii) **SI RG**: lime, P & K inputs, a new pasture variety, rotationally grazed, iii) **BAU RG**: rotationally grazed, no nutrient inputs, and iv) **SI SS**: lime, P & K inputs, set-stocked. Soil samples were taken from each treatment using a 25 mm auger to a depth of 15 cm (16 cores from four 30 × 30 m quadrats in each treatment plot). Cores were bulked into a single sample per quadrat (fresh mass ca. 500 g), giving a total of 16 samples from an original 64 samples.

Samples weighing 2 g were taken from each of the 16 samples across the four treatments described in Chapter 7, and were freeze dried and sieved. Samples were weighed in 10 ml glass tubes with Teflon inserts to ensure that no sample material escaped under heat treatments. A 7DHC spike was added before extraction for all soils, with a number of control replicates which received no 7DHC spike. Three ml of 0.05 KOH mL methanol was added to the samples, which were then wrapped in foil to prevent photochemical effects causing the sterols to decompose. Samples were placed for 12 hours in a shaking incubator at 100 rpm, room temperature pressure. The saponification step involved vortexing samples and then incubating them on an 80°C heating block for 30 minutes. Samples were partitioned three times using 2 ml petroleum ether (top layer pipetted into glass vial), and were then vortexed and centrifuge for 1 minute at 1000 RPM each time. Samples were reduced using N gas until dry. One ml of methanol was added to resuspend samples, which were then sonicated in a sonicating bath. A 0.2 µm filter syringe was used to filter the resuspended samples into a glass vial, and care was taken to push air through the syringe to remove all of the sample from the filter. Sterol concentrations were then measured using a HPLC Column (Agilent Technologies).

9.4. Results and discussion

Cholesterol, ergosterol, sistosterol and stigmasterol were measured across all 16 samples from all four treatments using the LCMS method (Figure 9.1). Cholesterol and sistosterol were the most abundant sterols detected in the samples measured. Of these, no significant differences were detected between overall sterol content of the samples, or between individual treatments. However, a significant combined effect of grazing regime and nutrient amendments was detected on sistosterol relative abundance (Two-way ANOVA, p = 0.02).

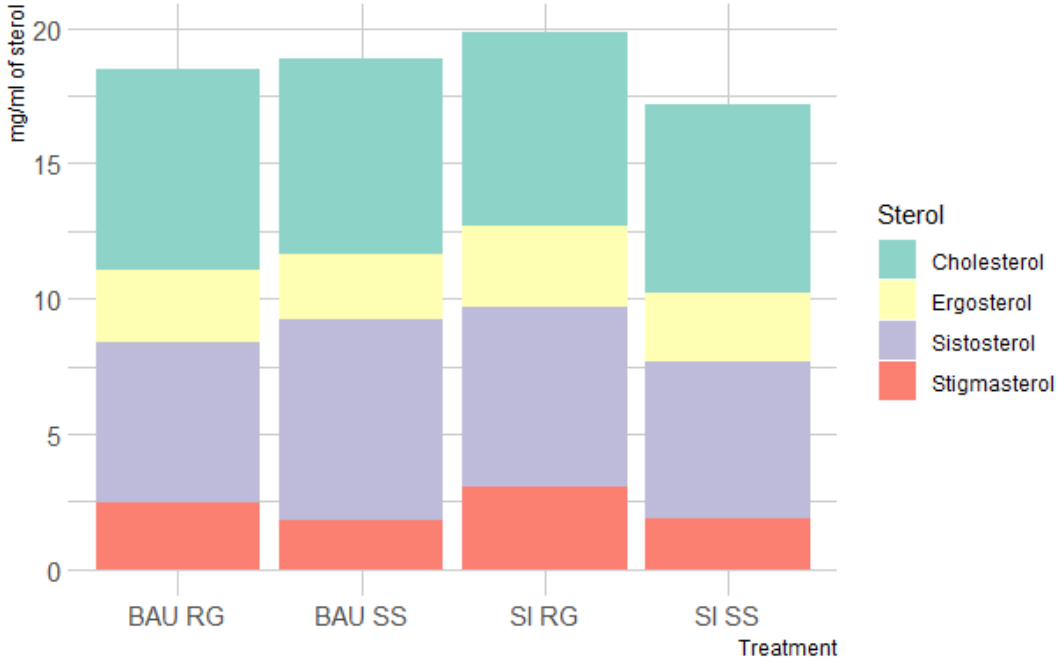

Figure 9.1: Stacked barchart showing log transformed volumes of the four sterols tested across all treatments (four samples in each treatment, mean of four quadrats)

Sitosterols are widely observed across the plant kingdom, and variation in concentrations under different treatments may reflect changes in vegetation composition and root proliferation. The fact that the effect of rotational grazing and nutrient amendments may have acted in combination suggests a more complex interaction between plants, the soil microbial community and management approach, and it is necessary that more research is undertaken across a larger number of samples in different treatments and soil types to confirm these observations.

It is now understood that variation in levels of sitosterol and stigmasterol during crop development may be linked to stress compensation (Aboobucker and Suza, 2019), due to sterols being used by plants to maintain plasma membrane fluidity via the phospholipids. Further research is necessary

over a longer time period to quantify variation in temporal levels of these sterols, which may afford insights into the relationship between sustainable intensification approaches and plant stress.

The method was unable to identify differences in fungal biomass (ergosterol) under different sustainable intensification approaches. Data presented in Chapters 3 and 4 suggested that substantial differences existed between fungal populations in under different treatments, and therefore further assays with a greater number of samples from these treatments will be necessary to properly investigate this possibility. The data observed from the sterols assay was noisy, and three extreme outliers were removed from the analyses. Other methodologies have suggested integrating a step to remove non-saponifiable lipids by washing the extracts with hexane (Weete and Ghandi 1997), and further investigation is necessary to determine whether this step affords clearer findings across soil isolates.

A major restriction of this method was that only four sterols were measured by the HPLC reader. Some studies have been able to ascertain as many as 18 sterols (Weete and Ghandi, 1997; Weete and Ghandi, 1999) specifically associated with fungi, and were able to suggest detailed conclusions about the sterols associated with a number of fungal taxa. The method described in this study requires some development before it can offer the level of detail needed to draw conclusions on fungal biomass levels of individual fungal taxa associated with various treatments.

One issue inherent with attempts to elucidate which soil processes are determined by microbial diversity is how to discern between active and dormant cells in extracellular DNA. One possible mechanism which could be used to provide insights on this is bio-orthogonal non-canonical amino acid tagging BONCAT, which has been successfully used to measure translationally active cells in soils by Couradeau *et al*. (2019), who revealed that the composition of soil active fraction can be distinct from the overall microbial population in extractable soils.

9.5. References

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Appendix 4

A TXRF method for elemental analysis

10.1. Materials and methods

All biostimulants used in this study were examined for elemental composition using a total reflection X-ray fluorescence method (TXRF), which can offer accurate measurements of major and trace elements within samples. Samples were first thoroughly ground and sieved (50 µm), and a 20 mg subsample was suspended in 1 ml of 1% Triton-X solution. Selenium was used as an internal standard due to its extremely rare presence in soils. 10 µl of 1000 µg ml^{-1} selenium standard was used, which gave an internal standard mass of 10 µg. After vortexing, 10 µl of the sample was transferred to a quartz glass sample plate (dimensions were 30 mm diameter, thickness = 3 mm) and dried on a heating plate. Sample plates were siliconized by applying 10 µl of a silicon solution in isopropanol to prevent the samples spreading across the sample plates. Measurements were performed using a S2 PICOFOX™ TXRF-spectrometer (Bruker AXS Microanalysis GmbH, Germany). Measurements were taken at a 50 kV operating voltage and a 750 µA current. The measurement period was 1000 s per sample. The treatment of the X-ray spectra and analysis of the fluorescence peak overlaps were performed using SPECTRA 6.1 software.

Appendix 5: *Chapters 4 and 5*

11.1. Ion Torrent phylum abundances – All samples

Table 11.1: Top 150 most abundant sequences across all treatments in Chapter 4

Phylum	Class	Order	Family	Genus (species if known)	Mean	KB-P	KB+P	KB(S)-P	KB(S)-P	UM(S)-P	UM(S)+P	UR(S)-P	UR(S)+P	UM-P	UM+P	UR-P	UR+P	Control+P
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella (parvispora)	24.63	4.42	9.79	4.20	16.23	3.49	76.59	3.49	34.34	9.56	27.83	18.59	69.77	26.70
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Pseudogymnoascus	9.90	26.33	13.41	14.25	17.34	21.96	1.20	8.05	6.19	20.40	4.86	3.44	2.26	13.41
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Sphaerosporella	9.74	12.30	25.73	19.50	7.59	14.65	0.00	50.90	0.00	0.00	0.00	19.25	4.95	0.00
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella (alpina)	8.12	3.15	1.88	2.07	1.43	1.40	2.10	3.69	16.90	8.59	16.97	22.26	0.63	14.69
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Eupenicillium	5.70	13.80	16.74	8.64	14.78	21.63	0.43	2.96	1.35	2.82	1.35	3.61	0.65	4.09
Ascomycota	Leotiomycetes	Onygenales	Onygenaceae	Chrysosporium	4.38	0.65	0.46	1.23	0.24	3.15	0.44	2.38	0.32	32.44	0.12	1.71	0.04	1.59
Basidiomycota	Tremellomycetes	Tremellales	Trimorphomycetaceae	Saitozyma	3.13	5.92	9.19	11.39	16.80	1.62	0.76	0.33	0.94	1.13	0.51	1.32	0.31	0.40
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	PenicilliumY	2.72	5.03	3.18	3.33	6.91	2.02	0.74	0.45	4.89	0.94	4.32	1.35	1.23	4.10
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	PenicilliumZ	2.51	1.19	1.31	2.53	1.62	3.80	2.36	1.73	4.30	1.28	0.93	1.77	0.40	2.47
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Chromocleista	2.52	0.59	1.73	0.48	1.86	1.20	6.04	0.32	3.48	0.87	2.43	1.77	5.76	4.62
Ascomycota	Leotiomycetes	Leotiomycetes incertae sedis	Myxotrichaceae	Myxotrichum	2.71	0.03	0.00	0.04	0.02	0.04	0.46	0.41	1.21	0.27	24.87	0.00	4.83	0.20
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	OTU 11	2.34	2.03	0.80	3.38	0.61	3.62	0.29	3.61	1.26	2.03	0.57	2.51	0.42	1.33
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	Oidiodendron	1.82	1.75	0.44	1.99	0.67	2.54	0.28	1.35	0.74	1.49	0.65	1.68	0.46	1.50
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella (alpina)	1.87	0.09	0.38	0.04	0.13	0.13	0.62	0.05	4.30	2.36	4.26	6.25	0.17	2.12
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	PolyphilusAFF	1.50	1.09	1.39	2.16	0.44	2.11	0.23	1.95	1.03	1.35	0.34	1.34	0.54	2.26
Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Phellopilus	1.09	1.45	0.12	6.90	0.03	1.66	0.00	0.70	0.31	0.35	0.00	1.22	0.01	1.03
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	0.75	2.29	2.43	0.75	2.99	0.74	1.20	0.27	0.64	0.24	0.24	0.34	0.08	0.46
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacinaceae unc.	0.82	0.61	0.33	0.64	0.22	1.31	0.20	1.18	0.81	1.06	0.31	0.49	0.12	0.77
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Hyaloscypha 19	0.85	0.19	0.34	0.33	0.49	0.76	0.58	0.44	3.47	0.45	0.65	0.43	0.25	1.25
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Hyaloscypha 20	0.72	0.61	0.45	0.92	0.29	0.91	0.18	1.07	1.09	0.51	0.40	0.66	0.15	0.75
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Meyerozyma (guilliermondii)	0.64	0.01	0.44	0.04	0.02	0.31	0.53	3.21	0.56	0.05	0.83	0.03	0.36	1.65
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella parvispora (OTU45)	0.59	0.04	0.13	0.07	0.39	0.09	1.39	0.09	0.57	0.19	0.52	0.31	2.26	1.12
Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	Solicoccozyma	0.40	1.32	0.73	0.80	0.66	0.34	0.08	0.31	0.19	0.16	0.23	0.32	0.07	0.34
Basidiomycota	Agaricomycetes	Geastrales	Sphaerobolaceae	Sphaerobolus	0.47	0.16	0.80	0.25	0.08	0.97	0.03	1.60	0.35	0.23	0.45	0.46	0.19	0.68
Fungi incertae sedis	Mucoromycotina	Mucorales	Umbelopsidaceae	Umbelopsis	0.29	1.90	0.89	0.85	1.75	0.33	0.01	0.03	0.05	0.03	0.06	0.09	0.03	0.08
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Mucor	0.22	2.78	0.40	1.46	1.01	0.06	0.02	0.00	0.00	0.00	0.04	0.07	0.00	0.00
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	OTU 79	0.40	0.37	0.18	0.57	0.15	0.62	0.04	0.69	0.21	0.33	0.08	0.40	0.07	0.29

X	X	X	X	OTU 25	0.31	0.54	0.63	0.35	0.15	1.15	0.10	0.10	0.31	0.00	0.00	0.77	0.08	0.56
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella elongata	0.32	0.58	0.03	0.40	0.03	0.21	0.02	0.87	0.34	0.02	0.03	0.06	0.01	1.77
X	X	X	X	OTU 32	0.18	2.05	0.02	1.66	0.08	0.02	0.00	0.01	0.00	0.02	0.00	0.33	0.00	0.03
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	0.28	0.58	0.23	0.06	0.03	0.08	0.69	0.07	0.10	0.00	1.52	0.74	0.03	0.02
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Exophiala	0.28	0.03	0.52	0.00	0.24	0.04	0.14	0.11	0.80	0.42	0.38	0.03	1.02	0.23
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Cadophora	0.27	0.34	0.07	0.33	0.00	0.01	0.16	0.09	0.49	0.39	0.16	0.06	0.14	0.24
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	Lecanicillium	0.29	0.04	0.13	0.02	0.03	0.21	0.31	0.08	0.00	1.63	0.06	0.00	0.04	1.09
X	X	X	X	OTU 31	0.25	0.28	0.00	0.17	0.00	0.00	0.13	0.07	0.04	0.00	0.00	0.47	0.00	0.00
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	OidiodendronX	0.23	0.13	0.13	0.14	0.15	0.22	0.06	0.16	0.46	0.16	0.38	0.08	0.06	0.39
Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	Coniochaeta	0.21	0.03	0.20	0.11	0.02	0.11	0.00	0.30	0.43	0.98	0.10	0.20	0.06	0.14
X	X	X	X	OTU 42	0.22	0.00	0.02	0.00	0.00	0.00	0.00	0.00	1.69	0.00	0.00	0.00	0.00	0.90
Basidiomycota	Agaricomycetes	Auriculariales	Exidiaceae	Basidiodendron	0.20	0.11	0.06	0.07	0.02	0.23	0.03	0.19	0.28	0.08	0.09	0.06	0.02	0.49
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 326	0.19	0.05	0.14	0.05	0.54	0.02	0.04	0.02	0.62	0.07	0.12	0.19	0.01	0.55
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 100	0.16	0.36	0.02	0.19	0.00	0.09	0.00	0.39	0.16	0.02	0.03	0.03	0.01	0.94
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacina	0.17	0.10	0.03	0.08	0.00	0.17	0.00	0.12	0.02	0.14	0.00	0.04	0.00	0.02
X	X	X	X	OTU 44	0.17	0.07	0.03	0.20	0.06	0.14	0.06	0.15	0.10	0.08	0.04	0.10	0.02	0.10
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Mucor	0.17	0.14	0.00	0.34	1.64	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Hypocrea	0.14	0.17	0.29	0.16	0.08	0.09	0.01	0.03	0.08	0.84	0.08	0.11	0.04	0.07
Ascomycota	Sordariomycetes	Ophiostomatales	Ophiostomataceae	Ophiostoma	0.16	0.00	0.05	0.00	0.00	0.01	0.06	0.05	0.03	0.32	0.49	0.03	0.84	0.14
Ascomycota	X	X	X	OTU 55	0.14	0.00	0.30	0.03	0.00	0.39	0.12	0.35	0.07	0.47	0.02	0.08	0.02	0.08
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	OTU 43	0.13	0.13	0.18	0.20	0.03	0.15	0.01	0.69	0.00	0.02	0.00	0.14	0.02	0.00
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	OTU 40	0.15	0.04	0.06	0.44	0.00	0.34	0.00	0.08	0.21	0.37	0.09	0.13	0.00	0.08
X	X	X	X	OTU 59	0.15	0.00	0.03	0.02	0.00	0.02	0.00	0.52	0.00	0.00	0.00	1.24	0.00	0.00
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 277	0.14	0.00	0.03	0.00	0.03	0.01	0.21	0.00	0.45	0.19	0.36	0.23	0.07	0.10
Basidiomycota	Microbotryomycetes	Microbotryomycetes incertae sedis	Microbotryomycetes incertae sedis	Kriegeria	0.13	0.08	0.05	0.22	0.08	0.15	0.05	0.13	0.07	0.09	0.06	0.10	0.03	0.09
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Amylomyces	0.14	0.00	0.00	0.05	0.00	0.21	0.00	0.00	1.17	0.00	0.00	0.00	0.00	0.11
X	X	X	X	OTU 80	0.11	0.10	0.02	0.15	0.07	0.15	0.09	0.03	0.03	0.08	0.05	0.15	0.02	0.14
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	OTU 57	0.11	0.05	0.02	0.49	0.00	0.15	0.00	0.02	0.00	0.02	0.00	0.00	0.00	0.00
Ascomycota	Leotiomycetes	Leotiomycetes incertae sedis	Myxotrichaceae	OTU 308	0.10	0.12	0.02	0.16	0.06	0.16	0.02	0.06	0.03	0.08	0.03	0.04	0.03	0.15
Ascomycota	Sordariomycetes	X	X	OTU 58	0.10	0.14	0.02	0.27	0.00	0.19	0.00	0.06	0.00	0.05	0.00	0.13	0.00	0.04

Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	OTU 114	0.06	0.33	0.12	0.11	0.28	0.21	0.00	0.00	0.00	0.02	0.03	0.02	0.02	0.03
Ascomycota	Leotiomycetes	X	X	OTU 68	0.09	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Davidiella	0.08	0.05	0.03	0.08	0.00	0.12	0.02	0.02	0.02	0.01	0.04	0.06	0.06	0.22
Ascomycota	X	X	X	OTU 65	0.08	0.00	0.11	0.04	0.02	0.07	0.05	0.07	0.11	0.12	0.14	0.05	0.03	0.20
Ascomycota	Sordariomycetes	X	X	OTU 72	0.08	0.05	0.02	0.20	0.00	0.06	0.02	0.15	0.02	0.04	0.00	0.07	0.01	0.05
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	OTU 229	0.06	0.04	0.23	0.12	0.00	0.06	0.00	0.32	0.00	0.00	0.00	0.21	0.02	0.00
Ascomycota	Sordariomycetes	X	X	OTU 81	0.06	0.12	0.13	0.07	0.65	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	Trechispora	0.06	0.08	0.03	0.03	0.00	0.00	0.01	0.03	0.02	0.04	0.00	0.02	0.01	0.04
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolales incertae sedis	Rhodotorula	0.07	0.01	0.00	0.40	0.00	0.12	0.00	0.17	0.04	0.04	0.02	0.05	0.01	0.00
Ascomycota	Leotiomycetes	X	X	OTU 517	0.07	0.03	0.04	0.03	0.00	0.08	0.00	0.02	0.18	0.20	0.04	0.03	0.01	0.18
Basidiomycota	Microbotryomycetes	X	X	OTU 84	0.05	0.09	0.05	0.08	0.10	0.06	0.01	0.01	0.02	0.04	0.04	0.04	0.00	0.02
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Dissophora	0.06	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.17	0.11	0.17	0.08	0.05	0.05
X	X	X	X	OTU 189	0.05	0.07	0.09	0.05	0.00	0.21	0.02	0.00	0.08	0.00	0.00	0.11	0.02	0.10
Ascomycota	Dothideomycetes	Capnodiales	X	OTU 96	0.06	0.02	0.03	0.03	0.04	0.02	0.04	0.01	0.08	0.08	0.08	0.05	0.01	0.14
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Calonectria	0.05	0.13	0.00	0.42	0.00	0.06	0.00	0.02	0.00	0.05	0.00	0.00	0.00	0.00
Chytridiomycota	Chytridiomycetes	Cladochytriales	Nowakowskiellaceae	Nowakowskiella	0.05	0.04	0.00	0.07	0.00	0.00	0.00	0.05	0.03	0.08	0.00	0.04	0.00	0.17
X	X	X	X	OTU 97	0.05	0.09	0.00	0.07	0.00	0.01	0.00	0.05	0.06	0.04	0.00	0.16	0.00	0.10
Fungi incertae sedis	Mucoromycotina	Mucorales	Cunninghamellaceae	Gongronella	0.01	0.35	0.17	0.02	0.13	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Basidiomycota	Cystobasidiomycetes	X	X	OTU 83	0.05	0.00	0.01	0.00	0.00	0.00	0.04	0.02	0.00	0.07	0.06	0.02	0.45	0.00
X	X	X	X	OTU 170	0.05	0.03	0.01	0.07	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.03	0.00	0.04
Ascomycota	X	X	X	OTU 78	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.51	0.00	0.00	0.00	0.00
Ascomycota	Leotiomycetes	X	X	OTU 171	0.05	0.00	0.02	0.03	0.03	0.06	0.01	0.05	0.13	0.03	0.09	0.00	0.00	0.10
Ascomycota	Leotiomycetes	Helotiales	X	OTU 109	0.05	0.06	0.03	0.13	0.00	0.08	0.00	0.05	0.00	0.03	0.00	0.00	0.00	0.00
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	OTU 164	0.05	0.04	0.03	0.08	0.05	0.09	0.02	0.04	0.07	0.05	0.02	0.02	0.01	0.05
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Sorocybe	0.04	0.03	0.06	0.02	0.00	0.07	0.02	0.05	0.04	0.10	0.04	0.00	0.04	0.07
X	X	X	X	OTU 132	0.05	0.04	0.04	0.06	0.00	0.04	0.00	0.09	0.03	0.05	0.03	0.07	0.00	0.14
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida (subhashii)	0.04	0.02	0.08	0.09	0.06	0.13	0.02	0.02	0.03	0.02	0.03	0.08	0.01	0.00
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	OTU 110	0.03	0.14	0.07	0.12	0.16	0.01	0.00	0.00	0.00	0.02	0.00	0.04	0.00	0.00
Basidiomycota	Cystobasidiomycetes	Cystobasidiomycetes incertae sedis	Cystobasidiomycetes incertae sedis	Cyrenella	0.04	0.00	0.01	0.00	0.00	0.04	0.02	0.06	0.13	0.06	0.15	0.02	0.02	0.00
Ascomycota	Leotiomycetes	Helotiales	X	OTU 489	0.04	0.06	0.00	0.11	0.00	0.08	0.00	0.06	0.00	0.03	0.00	0.03	0.00	0.02

Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	OTU 121	0.04	0.03	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.02	0.00	0.02
Ascomycota	Leotiomycetes	X	X	OTU 487	0.04	0.03	0.01	0.02	0.00	0.05	0.00	0.03	0.04	0.02	0.02	0.05	0.01	0.07
Basidiomycota	Cystobasidiomycetes	X	X	OTU 125	0.04	0.00	0.01	0.00	0.00	0.00	0.00	0.03	0.11	0.17	0.00	0.00	0.02	0.08
Basidiomycota	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	Cystobasidium	0.04	0.00	0.05	0.00	0.00	0.03	0.02	0.20	0.00	0.07	0.05	0.02	0.03	0.00
Ascomycota	X	X	X	OTU 89	0.04	0.05	0.01	0.11	0.00	0.06	0.00	0.09	0.00	0.03	0.00	0.00	0.00	0.02
X	X	X	X	OTU 94	0.03	0.09	0.01	0.07	0.00	0.04	0.00	0.01	0.06	0.03	0.02	0.06	0.00	0.08
Ascomycota	Sordariomycetes	X	X	OTU 249	0.02	0.17	0.00	0.12	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.03	0.00	0.02
Ascomycota	Dothideomycetes	Capnodiales	Capnodiales incertae sedis	OTU 117	0.04	0.01	0.03	0.04	0.03	0.03	0.05	0.02	0.05	0.01	0.04	0.03	0.02	0.04
Ascomycota	Leotiomycetes	X	X	OTU 450	0.03	0.05	0.02	0.04	0.03	0.05	0.00	0.00	0.04	0.13	0.02	0.01	0.01	0.05
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 490	0.04	0.00	0.02	0.02	0.00	0.02	0.11	0.00	0.05	0.02	0.04	0.03	0.12	0.03
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	0.04	0.00	0.00	0.08	0.00	0.00	0.00	0.03	0.00	0.04	0.00	0.00	0.00	0.00
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	0.04	0.00	0.00	0.20	0.00	0.03	0.00	0.04	0.00	0.02	0.00	0.00	0.00	0.00
X	X	X	X	OTU 172	0.03	0.02	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
Ascomycota	Leotiomycetes	X	X	OTU 444	0.03	0.02	0.01	0.03	0.00	0.04	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00
Ascomycota	Leotiomycetes	Helotiales	X	OTU 168	0.03	0.02	0.05	0.04	0.00	0.02	0.00	0.05	0.00	0.05	0.00	0.02	0.00	0.02
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Tolypocladium	0.03	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.02	0.00	0.00	0.11
Ascomycota	Sordariomycetes	X	X	OTU 129	0.03	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.18	0.00	0.00	0.00	0.03
X	X	X	X	OTU 120	0.03	0.01	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00
Fungi incertae sedis	Zoopagomycotina	Zoopagales	Piptocephalidaceae	Kuzuhaea	0.01	0.08	0.19	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ascomycota	Leotiomycetes	Helotiales	X	OTU 273	0.03	0.00	0.01	0.05	0.00	0.09	0.00	0.02	0.04	0.00	0.01	0.03	0.04	0.03
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 107	0.02	0.00	0.12	0.00	0.00	0.09	0.02	0.03	0.00	0.05	0.00	0.00	0.01	0.00
X	X	X	X	OTU 153	0.02	0.06	0.06	0.07	0.00	0.02	0.02	0.00	0.04	0.00	0.02	0.02	0.00	0.03
Ascomycota	Sordariomycetes	Sordariales	X	OTU 154	0.02	0.00	0.03	0.00	0.00	0.03	0.00	0.03	0.05	0.02	0.02	0.01	0.00	0.03
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Hypholoma	0.03	0.00	0.01	0.03	0.00	0.03	0.00	0.07	0.00	0.01	0.03	0.04	0.01	0.00
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	OTU 240	0.02	0.03	0.02	0.03	0.00	0.03	0.00	0.07	0.00	0.03	0.02	0.01	0.01	0.00
Ascomycota	Sordariomycetes	X	X	OTU 147	0.02	0.02	0.02	0.03	0.00	0.06	0.00	0.04	0.05	0.01	0.00	0.03	0.01	0.00
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	OTU 134	0.02	0.00	0.00	0.00	0.00	0.02	0.12	0.00	0.00	0.03	0.02	0.01	0.01	0.05
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	OTU 525	0.02	0.03	0.01	0.06	0.00	0.03	0.00	0.01	0.03	0.02	0.00	0.00	0.00	0.02
X	X	X	X	OTU 160	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.05	0.00	0.00
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Pseudonectria	0.02	0.03	0.00	0.11	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.00

Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Paecilomyces	0.02	0.00	0.01	0.02	0.02	0.00	0.00	0.02	0.00	0.08	0.00	0.00	0.00	0.08
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	OTU 623	0.02	0.03	0.02	0.03	0.05	0.02	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.04
X	X	X	X	OTU 150	0.01	0.00	0.12	0.00	0.00	0.00	0.00	0.11	0.02	0.00	0.00	0.00	0.00	0.00
Ascomycota	Leotiomycetes	Leotiomycetes incertae sedis	Myxotrichaceae	Myxotrichum	0.02	0.00	0.01	0.00	0.00	0.03	0.01	0.04	0.00	0.00	0.02	0.01	0.01	0.06
Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae	Immersiella	0.02	0.00	0.01	0.00	0.00	0.05	0.00	0.04	0.00	0.00	0.00	0.03	0.00	0.04
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	0.02	0.00	0.00	0.00	0.00	0.01	0.00	0.04	0.02	0.02	0.01	0.02	0.00	0.05
X	X	X	X	OTU 224	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Basidiomycota	Exobasidiomycetes	Malasseziales	Malasseziaceae	Malassezia	0.02	0.00	0.02	0.00	0.00	0.02	0.00	0.02	0.00	0.01	0.03	0.00	0.00	0.05
Basidiomycota	Agaricomycetes	X	X	OTU 175	0.01	0.06	0.00	0.05	0.00	0.01	0.00	0.03	0.02	0.00	0.00	0.00	0.00	0.04
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	OTU 268	0.01	0.02	0.06	0.00	0.00	0.01	0.00	0.05	0.00	0.00	0.00	0.06	0.00	0.00
Ascomycota	Leotiomycetes	Leotiomycetes incertae sedis	Myxotrichaceae	OTU 582	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.04	0.00

Table 11.2. Top 200 Sequences across all treatments in Chapter 5

Phylum	Class	Order	Family	Genus	Ecology	Mean	BAU SS	SI RG	BAU RG	SI SS
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 1		14.61	9.42	2.62	36.36	10.06
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus CU1	MR CHEG	12.85	11.34	8.22	5.01	26.82
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus CU4	MR CHEG	10.17	8.03	18.56	2.02	12.08
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe PS1	MR CHEG	5.47	3.66	9.74	5.61	2.86
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavulinopsis CPCO	MR CHEG	3.15	1.30	5.25	2.11	3.92
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Lepista	SAP SOIL	3.10	6.75	2.76	0.17	2.70
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavulinopsis CPLA	MR CHEG	2.85	4.19	1.50	5.17	0.53
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVFR	MR CHEG	2.61	2.46	6.74	0.08	1.17
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe HY2	MR CHEG	2.44	2.46	3.67	2.07	1.58
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Sorocybe	MR DSE	2.25	2.87	2.49	2.21	1.42
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Gliophorus	MR CHEG	2.23	4.76	1.55	2.22	0.41
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe HY5	MR CHEG	1.99	0.19	0.68	7.08	0.03
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVZO	MR CHEG	1.97	3.92	1.44	2.25	0.27
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavulinopsis CPFU	MR CHEG	1.46	3.16	0.20	2.35	0.12
Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	Solicoccozyma		1.45	1.62	1.94	0.92	1.31
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	SAP SOIL	1.35	1.59	1.74	0.49	1.58
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe PS4	MR CHEG	1.24	0.34	0.80	1.93	1.88
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus 3	MR AM	1.19	0.93	1.68	0.77	1.38
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	Trechispora	SAP SOIL	1.08	1.57	0.58	1.80	0.38
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVAC	MR CHEG	0.95	1.06	1.40	0.57	0.78

Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Pyrenochaeta	SAP	0.92	0.52	0.79	0.25	2.12
X	X	X	X	OTU 14		0.75	0.73	1.11	0.43	0.75
X	X	X	X	OTU 16		0.72	0.83	1.07	0.15	0.81
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	MR DSE	0.69	0.75	0.77	0.43	0.80
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Ramariopsis RMKU	MR CHEG	0.68	0.97	0.32	0.92	0.50
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVAR	MR CHEG	0.63	1.11	0.52	0.50	0.40
Ascomycota	Geoglossomycetes	Geoglossales	Geoglossaceae	Geoglossum	MR CHEG	0.59	1.12	0.38	0.47	0.38
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lachnum	MR DSE	0.58	0.57	0.61	0.21	0.91
Ascomycota	Leotiomycetes	Helotiales	X	OTU 47		0.49	0.59	0.49	0.62	0.29
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Nolanea	MR CHEG	0.43	0.22	0.93	0.38	0.20
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVIN	MR CHEG	0.42	0.12	0.92	0.22	0.44
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Ramariopsis RMPU	MR CHEG	0.42	0.54	0.25	0.74	0.15
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	MR DSE	0.41	0.29	0.50	0.27	0.58
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Panaeolus	SAP SOIL	0.40	0.84	0.19	0.45	0.14
Ascomycota	Geoglossomycetes	Geoglossales	Geoglossaceae	Trichoglossum	MR CHEG	0.38	0.02	0.11	0.18	1.20
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus CU2	MR CHEG	0.35	0.06	0.01	0.00	1.34
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 38		0.34	0.64	0.71	0.01	0.01
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Calyptella	SAP	0.33	0.11	0.90	0.02	0.28
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Melanotus	SAP	0.29	0.52	0.13	0.12	0.40
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 108		0.27	0.50	0.18	0.38	0.03
Ascomycota	X	X	X	OTU 56		0.26	0.28	0.22	0.11	0.41
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Porpolomopsis	MR CHEG	0.25	0.89	0.11	0.02	0.00
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 49		0.25	0.29	0.61	0.05	0.06
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	SAP	0.25	0.09	0.28	0.10	0.53

Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	MR AM	0.25	0.29	0.25	0.30	0.16
X	X	X	X	OTU 54		0.24	0.23	0.35	0.04	0.35
Ascomycota	X	X	X	OTU 77		0.24	0.27	0.32	0.23	0.14
Ascomycota	Leotiomycetes	X	X	OTU 74		0.24	0.13	0.21	0.19	0.40
Ascomycota	Dothideomycetes	X	X	OTU 59		0.23	0.48	0.14	0.19	0.13
Ascomycota	X	X	X	OTU 53		0.22	0.31	0.24	0.25	0.08
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 57		0.22	0.12	0.40	0.21	0.14
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Mycena	SAP SOIL	0.22	0.63	0.08	0.13	0.03
Basidiomycota	Tremellomycetes	Tremellales	Trimorphomycetaceae	Saitozyma		0.21	0.20	0.24	0.14	0.26
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Tetracladium	MR DSE	0.21	0.18	0.17	0.07	0.43
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	SAP DUNG	0.20	0.18	0.18	0.14	0.27
Basidiomycota	Microbotryomycetes	Microbotryomycetes incertae sedis	Microbotryomycetes incertae sedis	Kriegeria	PATH SMUT	0.19	0.25	0.27	0.05	0.20
Ascomycota	X	X	X	OTU 65		0.18	0.20	0.26	0.06	0.21
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Mollisia	MR DSE	0.18	0.18	0.16	0.14	0.23
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinellus	SAP SOIL	0.17	0.19	0.29	0.08	0.13
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVFL	MR CHEG	0.17	0.30	0.15	0.09	0.13
Ascomycota	Geoglossomycetes	Geoglossales	Geoglossaceae	Glutinoglossum	MR CHEG	0.16	0.21	0.13	0.22	0.10
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Microglossum	MR DSE	0.16	0.24	0.17	0.11	0.13
Basidiomycota	Agaricomycetes	X	X	OTU 70		0.15	0.17	0.24	0.06	0.14
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Cudoniella	MR DSE	0.15	0.20	0.15	0.13	0.13

Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	PATH	0.15	0.18	0.10	0.06	0.25
Basidiomycota	Agaricomycetes	X	X	OTU 63		0.15	0.00	0.01	0.39	0.19
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVGU	MR CHEG	0.14	0.14	0.10	0.12	0.19
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 67		0.14	0.25	0.09	0.13	0.08
Fungi incertae sedis	Mucoromycotina	Endogonales	Endogonaceae	Endogone		0.13	0.24	0.13	0.09	0.08
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	OTU 215		0.13	0.14	0.11	0.13	0.15
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	OTU 92	MR CHEG	0.12	0.00	0.00	0.00	0.48
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 79		0.12	0.17	0.18	0.04	0.09
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Camarophyllopsis CAMS	MR CHEG	0.12	0.07	0.05	0.33	0.02
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 76		0.12	0.00	0.00	0.25	0.22
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	Operculomyces		0.11	0.09	0.23	0.03	0.11
Chytridiomycota	Chytridiomycetes	Rhizophydiales	X	OTU 111		0.11	0.15	0.10	0.09	0.10
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Camarophyllopsis CAMH	MR CHEG	0.11	0.02	0.01	0.07	0.34
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 106		0.11	0.09	0.13	0.06	0.15
Ascomycota	Archaeorhizo-mycetes	Archaeorhizo-mycetales	Archaeorhizomycetales incertae sedis	Archaeorhizomyces		0.11	0.33	0.03	0.03	0.05
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavulinopsis CPX1	MR CHEG	0.11	0.07	0.04	0.29	0.04
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OTU 154		0.11	0.09	0.09	0.08	0.17
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 85		0.11	0.05	0.06	0.01	0.30

Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Calocybe	SAP SOIL	0.10	0.00	0.00	0.12	0.29
Ascomycota	Leotiomycetes	Helotiales	X	OTU 121		0.10	0.14	0.05	0.12	0.09
Basidiomycota	Agaricomycetes	X	X	OTU 115		0.10	0.08	0.08	0.09	0.14
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Hypholoma	SAP WRF	0.10	0.26	0.05	0.04	0.04
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Tubaria	SAP	0.09	0.09	0.02	0.03	0.22
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Cochliobolus	PATH	0.09	0.03	0.12	0.03	0.18
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	OTU 147		0.08	0.09	0.10	0.08	0.07
Ascomycota	Leotiomycetes	Helotiales	X	OTU 162		0.08	0.08	0.07	0.08	0.11
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 182		0.08	0.08	0.13	0.01	0.10
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Alphamycetaceae	Betamyces		0.08	0.08	0.10	0.04	0.10
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	OTU 1640	MR CHEG	0.08	0.05	0.04	0.03	0.20
Ascomycota	X	X	X	OTU 103		0.08	0.15	0.04	0.01	0.10
Ascomycota	Pezizomycetes	Pezizales	Ascodesmidaceae	Ascodesmis	SAP DUNG	0.08	0.05	0.15	0.00	0.10
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 849		0.08	0.13	0.12	0.00	0.05
Pseudofungi	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia		0.07	0.09	0.10	0.01	0.10
Blastocladiomycota	Blastocladiomycetes	Blastocladiales	Physodermataceae	Physoderma	PATH PLANT	0.07	0.09	0.15	0.01	0.05
Ascomycota	Eurotiomycetes	X	X	OTU 126		0.07	0.04	0.12	0.07	0.05
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Arrhenia	MR CHEG	0.07	0.20	0.00	0.05	0.04
Pseudofungi	Oomycetes	Pythiales	Pythiaceae	Pythium F		0.07	0.04	0.11	0.01	0.12
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Flagelloscypha	SAP	0.07	0.05	0.05	0.03	0.15
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	SAP SOIL	0.07	0.06	0.14	0.00	0.06

Ascomycota	Leotiomycetes	Helotiales	X	OTU 163		0.06	0.10	0.04	0.07	0.05
Basidiomycota	Agaricomycetes	X	X	OTU 116		0.06	0.05	0.12	0.00	0.08
Ascomycota	Archaeorhizomycetes	Taphrinomycotina	X	OTU 119		0.06	0.12	0.05	0.01	0.08
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 719		0.06	0.03	0.08	0.01	0.14
Ascomycota	X	X	X	OTU 110		0.06	0.09	0.03	0.05	0.10
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeodothis	PATH	0.06	0.05	0.06	0.04	0.10
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe PS3	MR CHEG	0.06	0.04	0.08	0.13	0.00
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	OTU 229		0.06	0.13	0.05	0.03	0.04
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacina	MR EM	0.06	0.09	0.03	0.10	0.03
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Ramariopsis RMCR	MR CHEG	0.06	0.01	0.03	0.05	0.15
X	X	X	X	OTU 141		0.06	0.07	0.07	0.03	0.06
X	X	X	X	OTU 132		0.06	0.05	0.09	0.02	0.06
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 122	YEAST	0.06	0.03	0.09	0.02	0.09
X	X	X	X	OTU 131		0.06	0.04	0.08	0.04	0.06
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	SAP SOIL	0.05	0.02	0.12	0.00	0.07
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus CU3	MR CHEG	0.05	0.03	0.00	0.00	0.19
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Rickenella	PATH BRYO	0.05	0.02	0.04	0.11	0.04
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 130		0.05	0.06	0.09	0.01	0.05
Ascomycota	Dothideomycetes	Pleosporales	Lentitheciaceae	Keissleriella	SAP	0.05	0.04	0.05	0.01	0.10
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 140		0.05	0.05	0.07	0.01	0.07
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	OTU 2199	MR CHEG	0.05	0.00	0.13	0.02	0.04

X	X	X	X	OTU 134		0.05	0.05	0.04	0.05	0.05
Ascomycota	X	X	X	OTU 225		0.05	0.02	0.05	0.10	0.01
Pseudofungi	Oomycetes	Pythiales	Pythiaceae	Pythium B		0.04	0.02	0.06	0.00	0.09
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 1234		0.04	0.11	0.00	0.06	0.00
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Ceratobasidiaceae unc.	MR EM	0.04	0.06	0.03	0.04	0.04
Ascomycota	X	X	X	OTU 160		0.04	0.05	0.06	0.02	0.04
Ascomycota	Dothideomycetes	Hysteriales	Hysteriaceae	Ostreichnion	SAP	0.04	0.05	0.05	0.04	0.02
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 242		0.04	0.08	0.02	0.04	0.01
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 329		0.04	0.05	0.05	0.02	0.04
Ascomycota	Leotiomycetes	X	X	OTU 241		0.04	0.04	0.08	0.01	0.01
Basidiomycota	Agaricomycetes	X	X	OTU 198		0.04	0.03	0.03	0.01	0.08
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellales incertae sedis	Modicella		0.04	0.01	0.13	0.01	0.00
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	OTU 193		0.04	0.04	0.04	0.02	0.05
X	X	X	X	OTU 161		0.04	0.03	0.11	0.00	0.00
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 176	MR CHEG	0.04	0.05	0.04	0.02	0.02
Ascomycota	X	X	X	OTU 188		0.04	0.04	0.04	0.01	0.06
Ascomycota	Leotiomycetes	Helotiales	X	OTU 187		0.03	0.05	0.04	0.01	0.03
Ascomycota	Leotiomycetes	X	X	OTU 1786		0.03	0.05	0.05	0.02	0.03
Ascomycota	X	X	X	OTU 148		0.03	0.02	0.05	0.06	0.01
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Entoloma	MR CHEG	0.03	0.00	0.00	0.13	0.00

Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 166		0.03	0.05	0.07	0.01	0.01
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVX2	MR CHEG	0.03	0.09	0.02	0.02	0.00
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria CVNZ	MR CHEG	0.03	0.09	0.01	0.03	0.00
Ascomycota	X	X	X	OTU 158		0.03	0.07	0.00	0.05	0.01
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 165		0.03	0.00	0.00	0.09	0.03
Glomeromycota	Glomeromycetes	Paraglomerales	Paraglomeraceae	Paraglomus	MR AM	0.03	0.04	0.04	0.01	0.04
Basidiomycota	Agaricomycetes	X	X	OTU 853		0.03	0.02	0.07	0.01	0.02
Ascomycota	X	X	X	OTU 233		0.03	0.04	0.03	0.02	0.04
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 1047		0.03	0.02	0.00	0.07	0.04
Ascomycota	Leotiomycetes	Helotiales	X	OTU 255		0.03	0.03	0.04	0.02	0.03
Pseudofungi	Oomycetes	Oomycetes IS	Oomycetes IS	Haptoglossa		0.03	0.03	0.03	0.02	0.04
Basidiomycota	Agaricomycetes	Polyporales	X	OTU 192		0.03	0.02	0.05	0.00	0.05
X	X	X	X	OTU 194		0.03	0.04	0.03	0.03	0.02
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 1233		0.03	0.02	0.04	0.03	0.04
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	OTU 156		0.03	0.02	0.00	0.09	0.00
Chytridiomycota	Chytridiomycetes	X	X	OTU 204		0.03	0.04	0.03	0.02	0.02
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 279		0.03	0.04	0.02	0.03	0.02
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus 4	MR AM	0.03	0.02	0.05	0.00	0.04
Pseudofungi	Oomycetes	Oomycetes IS	Lagenaceae	Lagena		0.03	0.02	0.03	0.03	0.04
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	OTU 231		0.03	0.01	0.03	0.05	0.02
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 190		0.03	0.07	0.03	0.01	0.00
Ascomycota	Leotiomycetes	Helotiales	X	OTU 173		0.03	0.02	0.03	0.04	0.03

Ascomycota	X	X	X	OTU 179		0.03	0.11	0.01	0.00	0.00
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Graddonia	MR DSE	0.03	0.04	0.01	0.02	0.04
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Gamsiella		0.03	0.03	0.03	0.01	0.04
X	X	X	X	OTU 181		0.03	0.00	0.00	0.00	0.11
Chytridiomycota	Chytridiomycetes	X	X	OTU 202		0.03	0.01	0.05	0.02	0.03
Ascomycota	Leotiomycetes	Helotiales	X	OTU 775		0.03	0.02	0.05	0.00	0.04
X	X	X	X	OTU 244		0.03	0.02	0.02	0.03	0.03
X	X	X	X	OTU 356		0.03	0.04	0.03	0.02	0.01
X	X	X	X	OTU 266		0.03	0.03	0.03	0.01	0.04
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	OTU 262		0.03	0.03	0.03	0.01	0.03
Ascomycota	X	X	X	OTU 205		0.03	0.05	0.03	0.01	0.01
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 1213		0.03	0.01	0.01	0.04	0.04
Basidiomycota	Agaricomycetes	X	X	OTU 159		0.03	0.02	0.00	0.07	0.01
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 1987		0.03	0.03	0.04	0.01	0.03
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	Melanomma	LICHEN	0.02	0.01	0.04	0.01	0.05
Ascomycota	Leotiomycetes	Helotiales	X	OTU 171		0.02	0.02	0.01	0.04	0.03
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 880		0.02	0.02	0.00	0.07	0.01
Ascomycota	X	X	X	OTU 238		0.02	0.04	0.03	0.01	0.02
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Pilaira		0.02	0.01	0.02	0.00	0.06
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Cladophialophora	MR DSE	0.02	0.05	0.03	0.02	0.00
Basidiomycota	Agaricomycetes	X	X	OTU 230		0.02	0.03	0.05	0.00	0.01

Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Massariosphaeria	SAP AQ	0.02	0.01	0.02	0.00	0.06
X	X	X	X	OTU 174		0.02	0.03	0.02	0.03	0.01
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 340		0.02	0.03	0.03	0.00	0.03
Basidiomycota	Agaricomycetes	X	X	OTU 222		0.02	0.02	0.02	0.04	0.02
Pseudofungi	Oomycetes	Pythiales	Pythiaceae	Pythium A		0.02	0.01	0.05	0.00	0.03
Ascomycota	X	X	X	OTU 209		0.02	0.04	0.05	0.00	0.00
Basidiomycota	Exobasidiomycetes	Entylomatales	Entylomataceae	Entyloma	PATH SMUT	0.02	0.02	0.03	0.01	0.03
Ascomycota	X	X	X	OTU 218		0.02	0.05	0.00	0.00	0.03
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 1805		0.02	0.01	0.00	0.06	0.02
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium		0.02	0.00	0.02	0.01	0.05
Ascomycota	X	X	X	OTU 280		0.02	0.04	0.01	0.02	0.01
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 536		0.02	0.03	0.02	0.01	0.03
Basidiomycota	Agaricomycetes	Auriculariales	Exidiaceae	Heterochaete	SAP WRF	0.02	0.06	0.00	0.01	0.00