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Increasing agricultural grass production using novel bio-inoculants

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Increasing agricultural grass production using novel bio-inoculants

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SUMMARY

Soil micro-organisms are a fundamental component of soil ecosystem services. Plant yields have shown to be increased through processes mediated by fungi, such as increased acquisition of important plant nutrients e.g. phosphate (P) and nitrogen (N). This has seen micro-organisms exploited commercially to create bio-inoculants (BIs). However, it is remarkably difficult to determine the effectiveness of commercially-available BIs that claim to promote crop yields as in most cases the underlying mechanisms responsible for these beneficial effects are unknown. The aims of the thesis were to examine, both within the laboratory and field, the efficacy of some commercially available BIs. Focusing on mechanisms of increased P acquisition mediated by the application of BIs. The effects of soil-P availability on BI performance were also explored. Whilst next generation DNA sequencing was utilised to explore changes in soil fungal assemblages after the introduction of BIs. As illustrated in Chapter 2, there exists much variability in efficacy testing and there is a distinct lack of robust field-based testing of commercial BIs. Chapter 3 consisted of two laboratory-based pot-trials; the first investigated the efficacy of five commercial BIs on *Lolium perenne* growth, the second explored the effect of an inert carrier media utilised in BI manufacture. Results found that all tested BIs increased grass yields significantly, and while many BIs contain non-living additives, treatments with living microbial fractions were found to have significantly more roots, leading to increased growth per unit P taken up by the grass. The second bioassay found the dual application of carrier media and mycorrhizal spores significantly increased grass yield, the inert carrier media a significant factor with respect to mycorrhizal root colonisation, increasing from 20% to 36%. Chapter 4 explored the phosphate solubilisation and mineralisation potential of the BI products. All BIs successfully mobilised P from recalcitrant P sources (ranging from 164 - 490 mg l^{-1}, for inorganic-P and 0 - 39 mg l^{-1} for organic-P). A pot trial investigated the phosphorus efficiency ratio of *Lolium perenne* following application of two inorganic fertilisers of varying solubility, triple super phosphate and rock phosphate. Two of the tested BIs were found to exhibit P mediated growth gains in the form of increased yield, total shoot P, and phosphorous efficiency ratio. Yield and shoot P gains were found to be mediated by differing fractions of the living component of each BI dependent on P source. Chapters 5 and 6 were field trials exploring BI effectiveness on sites of varying P availability. Chapter 5 was on a soil of adequate P (21 mg kg^{-1}). Overall the studies found limited benefits of BI application. Chapter 6 consisted of two field trials, one with a range of P availability (3.3 - 32 mg kg^{-1}), the second with low P availability (8 mg kg^{-1}). Results of the first found positive correlations with the % P and total forage P with increasing plant-available soil-P of BI-treated grass, but no significant increases in yield. The increased P acquisition and lack of yield would suggest that yield limitation was not driven by P. Similarly the results of the second trial found that while some of the BI treated grass yielded moderately higher than controls, there were no significant treatment effects on % P and total forage P, again suggesting yield gains were due to other factors. All BI treatments found to increase the N content of the forage compared to control. Chapter 7 examined the effects of various management practices on soil fungal abundances. Using DNA sequencing, fungal assemblages were found to be significantly affected by both treatment (BI application and N fertiliser), soil type and sampling date. N fertiliser was the only treatment to significantly affect fungal diversity and equitability measures. The study was able to show the potential of NGS technology, Ion Torrent™, for examining changes in fungal communities within the field. The results suggest soils with adequate levels of plant-available P may not see much benefit to warrant the application of BIs, or at least at the application rates recommended. Overall, much work is still required both within science and industry in the development and manufacture of bio-inoculants as a reliable method to increase crop yields.

ACKNOWLEDGMENTS

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ACRONYMS

AEC - Anion exchange capacity

ADP - Adenosine diphosphate

AM - Arbuscular mycorrhiza

ATP - Adenosine triphosphate

ATM - Atmospheric deposition

BCa - Bias-corrected and accelerated

BG - Biagro® Grass

BI - Bio-inoculant

BMP - Biagro® MP

BS - Biagro® S

CEC - Cation exchange capacity

DAP - Di-ammonium phosphate

DI - De-ionised

DNA - Deoxyribonucleic acid

DM - Dry matter

DMY - Dry matter yield

EC - Electrical conductivity

EM - Effective micro-organisms®

EcM - Ecto-Mycorrhiza

ETOH - Ethanol

F% - Mycorrhizal frequency

GA - Gadlas

GIS - Geographical information systems

HCl - Hydrochloric acid

INVAM International Culture Collection of Arbuscular Mycorrhiza

KOH - Potassium hydroxide

LSD - Least significant difference

M% - Mycorrhizal intensity

MG - Morfa Ganol

MF - Mycorrhizal fungi

NO_3^- - Nitrate ion

NH_4^+ - Ammonium ion

OM - Organic matter

P - Phosphorus / Phosphate ion

PER - Phosphate efficiency ratio

P_i - Inorganic phosphate

PN - PhosN

P_o - Organic phosphate

PAR - Photosynthetically active radiation

PGPM - Plant growth promoting micro-organism

PB - Pikovskaya's broth

PMM - Phosphate mobilising micro-organism

RAF - Root associated fungi

RNA - Ribonucleic acid

RL - Root length

RLD - Root length density

RP - Rock phosphate

SA - Surface area

SRL	-	Specific root length
SSI	-	Single species inoculum
TCP	-	Tri-calcium phosphate
TRC	-	Total root length colonised
TSP	-	Triple super phosphate
TXRF	-	Total x-ray fluorescence
WHC	-	Water holding capacity

Units

Gg	-	Gigagram
ha	-	Hectare
kt	-	Kiloton
meq	-	Milliequivalent
mg	-	Milligram
mol	-	Mole
Mt	-	Megaton
pK_a	-	Acid disassociation constant
Tg	-	Teragram
µmol	-	Micromol
µS	-	Microsiemen
ybp	-	Years before present

DECLARATION

This work has not previously been accepted in substance for any degree, and is not concurrently submitted elsewhere in candidature for any degree.

Signed Candidate

Date Darren Owen

Statement 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged, giving explicit references. A reference list is appended at the end of each chapter.

Signed Candidate

Date Darren Owen

Statement 2

I agree to deposit an electronic copy of my thesis (the Work) in the Bangor University (BU) Institutional Digital Repository, the British Library ETHOS system, and/or in any other repository authorized for use by Bangor University and where necessary have gained the required permissions for the use of third party material.

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Date Darren Owen

CHAPTER 1: INTRODUCTION

1.1. General introduction

This study was funded by a Knowledge Economy Skills Scholarship (KESS). KESS is a major European convergence programme, led by Bangor University, with industry to support post-graduate research. Industry support for this research was from the Glenside group. Founded in 1982, Glenside has been working with livestock and arable farmers to improve the use of farm resources, helping to increase farming productivity throughout the UK.

The aims of the work undertaken within this thesis were to examine the efficacy of commercial biological inoculants (BIs), and explore the processes of grass growth yield enhancment micro-organisms found within many BIs, such as mycorrhizal fungi, utilise e.g. from the stimulation of root growth to increasing plant access to beneficial nutrients such as phosphorus (P).

The integration of BIs within integrated nutrient management aims to reduce inorganic fertiliser inputs by helping to exploit existing soil phosphate reserves. The market for biological products is rapidly expanding, due in no small part to the finite status of rock phosphate (RP), the base raw material of inorganic phosphate fertiliser manufacture.

Soil micro-organisms are a fundamental component of soil ecosystem services, facilitating the decomposition of organic wastes and crop residues (Javaid 2009), to nutrient cycling enhancement (Barea, Azcon & Azcon-Aguilar 2002). However, it is remarkably difficult to determine the effectiveness of commercially available BIs, which are claimed to promote plant growth; dozens of micro-organisms, used alone and in combination, are claimed to promote crop yields but in most cases the underlying mechanisms responsible for these beneficial effects are unknown.

There is a distinct lack of robust field-based testing of commercial BIs; the majority of studies focusing on laboratory-based pot trials. It has been suggested that experimental design is often not sufficiently rigorous to provide the statistically-validated evidence to support a direct beneficial effect from plant growth-promoting micro-organisms (Jones, Oburger 2011).

The efficacy of any fungal or bacterial strain utilised within inoculants is also subject to numerous soil, crop and environmental factors, from crop species compatibility, size and effectiveness of indigenous microbial populations, soil fertility and management (Adholeya, Tiwari & Singh 2005), to priority effects, in which initial populations of species determine final community composition (Mummey, Antunes & Rillig 2009). Collectively, these affect the soil microbial dynamics, functional processes and hence performance of commercial BIs.

1.2. Plan of thesis

Hereafter, the thesis is divided into seven chapters, starting with a review of existing research of BIs. In particular, this chapter reviews composition and function of BIs and the many factors which impact on their efficacy for increasing phosphorus availability in different soil and plant environments. A clarification of the nomenclature of BI classification is also proposed.

The experimental work is presented as five separate scientific papers; therefore some repetition of introductory material, methodology and references occurs but is unavoidable when preparing a thesis of this type.

The first two experimental chapters (Article I - II, Chapter 3 - 4) were laboratory based growth trials exploring the efficacy of five commercial BIs on *Lolium perenne* growth. Many BI products contain inert carriers, to aid dispersion and / or prolong shelf life. Chapter 3 also examined the effect of an inert carrier media, zeolite and attapulgite, with respect to mycorrhizal colonisation. Chapter 4 focused on grass growth with respect to P, interactive effects of BIs, rock phosphate and triple super phosphate were assessed in a laboratory based pot trial. A solubilisation investigation was also ran to assess the mobilisation of P from two recalcitrant P sources, tri-calcium phosphate and phytate, by the BIs.

Chapter 5 and 6 (Article III – IV) assessed the efficacy the BIs within the field. Three field trials were established each of varying plant-available soil P. Chapter 5 was on a typical agricultural soil with a medium amount of plant-available P. Chapter 6 consisted of two field trials, the first was on a site characterised with a heterogeneous distribution of available P, and investigated the effects of increased soil P on BI-treated grass. The second field trial, with a low amount of plant-available P, assessed the interactive effects of BIs and phosphate fertilisers, triple super phosphate and rock phosphate on yields of grass.

Chapter 7 (Article V) utilised next generation DNA sequencing to assess changes in fungal community assemblages of the three field trials (Chapter 5 & 6). Fungal DNA was extracted from soils of the three field trials and sequenced using the Ion Torrent^{TM} sequencing platform. Changes in fungal communities between sites and across time (July and September), and the effects of BI application and inorganic fertiliser application were assessed.

Chapter 8 includes a general discussion of results from all previous experimental chapters. Conclusions are drawn and areas of further work identified. Appendices include soil analysis procedures and supporting data results for each research chapter.

1.3. Aims and objectives

The main experimental research aims are:

- To examine the efficacy of some commercially available BIs in both lab and field based trials
- To investigate some of the BI mediated mechanisms used to increase plant-available P
- To explore the effects of varying P-indices, within the field, on BI performance
- To explore the changes in native fungal assemblages with the introduction of BIs, using next generation DNA sequencing
- To investigate the effects of inorganic fertilisers (N and P) on soil fungal communities

1.4. References

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CHAPTER 2: LITERATURE REVIEW

Use of commercial bio-inoculants to increase agricultural production through improved phosphorus acquisition

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2.1. Abstract

Meeting increasing global demand for food, fibre, and bioenergy requires efficient use of finite resources, and presents a key sustainability challenge to the agricultural industry, scientists and policy-makers. Increased interest in low-input agriculture in recent years has seen the growing development and use of commercial biological inoculants (bacteria and/or fungi) to increase the mobilisation of key nutrients, especially phosphorus (P), and enhance their availability to crop plants. Here, we review the terminology, composition and function of bio-inoculants and the many factors which impact on their efficacy for increasing P availability in different soil and plant environments. We conclude that the beneficial attributes of commercial bio-inoculants for integrated production systems are not clearly defined. Evidence to support their effectiveness is currently confounded by inadequate quality standards and insufficient knowledge of the underlying mechanisms, which have led to contradicting reports on field performance. There is, however, scope to engineer specific inoculant formulae for more sustainable P management in different system-soil-plant combinations, provided future research is properly structured to help understand the complexity and dynamism of microbial functioning and interactions in soils.

2.2. Introduction

The integration of biological inoculants (hereon referred to as 'bio-inoculants' (BIs)) within integrated nutrient management aims to reduce inorganic fertiliser inputs by helping to exploit legacy P reserves. The benefits of soil micro-organisms on soil ecosystem services are well documented and include the fixation of atmospheric N_2, decomposition of organic wastes and crop residues (Javaid 2009), pathogen suppression (Azcon-Aguilar, Barea 1996), nutrient cycling enhancement (Barea, Azcon & Azcon-Aguilar 2002), improved soil aggregation (Rillig, Mummey 2006), detoxifying pesticides and the production of bioactive compounds (Singh, Pandey & Singh 2011); all of which contribute to improving soil quality. The global market for BIs is growing at an estimated rate of ~ 10% per annum (Berg 2009); valued at $440 million in 2012 and expected to reach $1,295 million by 2020 (Transparency Market Research 2014). Demand is primarily driven from Asia, where governments, such as China and India, are promoting the use of BIs through tax incentives, tax exemptions and grants to provide support for their manufacture and distribution. However, it is remarkably difficult to determine the effectiveness of commercial BIs which are claimed to promote plant growth; dozens of micro-organisms, used alone and in combination, are claimed to promote crop yields but in most cases the underlying mechanisms responsible for these beneficial effects are unknown. Furthermore, quality control procedures within the industry and accepted standards to allow product comparison etc. are generally lacking. It is therefore timelier than ever to elucidate the effectiveness and mode of action of BIs.

Here we review the potential benefits of using commercial BIs designed to promote plant growth, with an emphasis on P supply through the exploitation of legacy P reserves. We provide a clarification of the nomenclature and classification of BIs within the broader plethora of terms used to describe "bio-fertiliser"-type products. Finally, we examine the effectiveness of commercially available BIs on both grass and cereal production, which account for the overwhelming proportion of land used for agricultural purposes.

2.2.1. Phosphorus

The principle role of P in plants is as a major constituent of DNA, RNA and phospholipids and the transport of energy within cells (ADP, ATP) and makes up ~ 0.2% of plant dry weight (Schachtman, Reid & Ayling 1998). P has a role in metabolism of carbohydrates and in fruit

setting, and moves at the end of the growing season to storage organs within the plant. There are three main differences between P and the other major biogeochemical elements (C, N, S, O, and H). Firstly, P does not exist in any significant quantities in nature in the gaseous state (as Phosphine, PH_3), and whilst small quantities of particulate and dissolved phosphates can be transported atmospherically, the atmosphere plays a minor role in the global P cycle (Jahnke 2000). Secondly, whilst within the laboratory several oxidation states are possible, in the natural environment oxidation-reduction does not govern the overall reactivity and distribution of P; within the natural systems P exists almost exclusively in the +V oxidation state as the tetrahedral oxy-anion, phosphate (PO_4^{3-}). Therefore the biogeochemical cycling of P within the natural environment involves a complexed, or slightly modified, form of the phosphate ion (Jahnke 2000). Thirdly, P is dominated by a single isotopic form (^{31}P) and only a very small fraction is made up of seven radioactive isotopes, of which two are commonly utilised in soil-plant studies ^{32}P and ^{33}P (produced in the atmosphere by nuclear reactions with argon). Both isotopes have very short half-lives (14.3 days for ^{32}P, 25.3 days ^{33}P) which accounts for both low activity and concentration within the environment (Frossard et al. 2009). The phosphate anion occurs in primary minerals, predominantly apatite; all apatite minerals contain the phosphate oxyanion linked by Ca^{2+} cations forming a hexagonal framework (Filippelli 2008), with each corner of the hexagonal cell bearing a different element ($Ca_5(PO_4)_2(F,Cl,OH)$)[1] of which fluorapatite is the most common species. P also exists as organophosphates (esters of phosphoric acid) found in DNA and RNA for example; and as phosphonates e.g. 2-aminoethylphosphonic acid, found within the cellular membranes of plants and some animals. From here on, P is used in reference to the phosphate anion (PO_4^{-3}).

The cycling of P is an important earth system process. It involves a continuous cycling of the element between land and ocean sediments (Fig. 2.1). Weathering and erosion mobilise P for delivery to the oceans, and riverine inorganic and organic P is transported in a continuum of physical forms which we represent as particulate and dissolved. Once the P enters the ocean it mostly remains chemically unchanged due to the low dissolution rates of the ocean (Filippelli 2008). Insoluble deposits accumulate on continental shelves, after many millennia, subduction raises the crustal plates and exposes the P deposits and the geochemical cycle begins again (Fig. 2.1). Human activities have doubled total river output of P, which whilst has a negative environmental cost, e.g. eutrophication, is also an inefficient use of a finite resource.

[1] Other minerals include monazite $(Ce,La,Nd,Th,Sm)(PO_4,SiO_4)$ and xenotime (YPO_4)

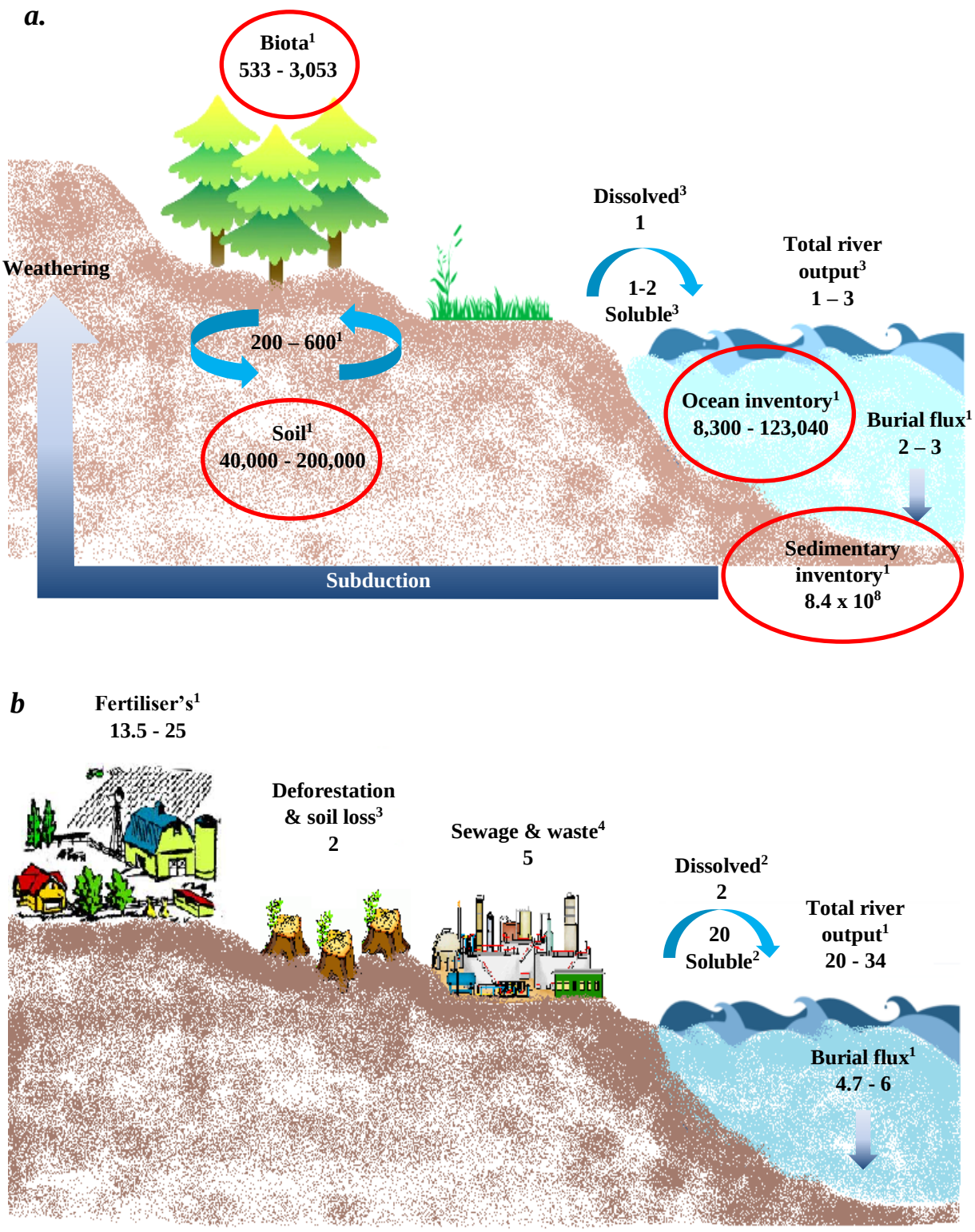

Figure 2.1. Global phosphorus cycling, riverine transport of P has increased substantially due to human activity, and as such led to eutrophication whilst the geological timescales involved in the formation of available phosphate reserves, render it a non-renewable resource (Ashley, Cordell & Mavinic 2011). *a*) Pre-anthropogenic *b*) Current, dissolved phosphorus cycle. Reservoirs circled red (in Tg P) fluxes denoted by arrows (Tg P year^{-1}) in the P mass balance. Figures adapted from [1] Penuelas et al (2013), [2]Smil (2000), [3]Filippelli (2008), [4]Withers et al. 2015

2.2.2. Phosphate reserves

P makes up ~ 0.7% of the earths' crust, making it the eleventh most abundant element (Schwedt 2001). It is an essential element for plant growth and hence is widely applied as inorganic fertiliser for agricultural purposes; global P fertiliser consumption for 2010 was approximately 37.6 Mt and is expected to increase to 42.3 Mt in 2014, with an annual 3% increase in demand thereafter (Heffer, Prud'homme 2010). Reserves of mineable rock phosphate (RP), which provide the base raw material for inorganic fertiliser production, are however relatively small and finite (current global reserves estimated at ~ 260 billion tonnes, mostly in North Africa) and may only last for 100-400 years (Steen 1998, Van Kauwenbergh 2010, Cordell, White 2011). Over 80% of RP reserves are utilised for fertiliser production since RP is the only source from which fertilisers can be made in large quantities. Future scarcity of RP may threaten future global food security, particularly so in areas which do not have any RP reserves (e.g. most of Europe). As the economic exploitation of RP becomes more difficult, the cost of P fertilisers will also increase, putting further pressure on agricultural profitability and rural livelihoods (Elser et al. 2014).

This potential crisis is exacerbated by the increased agricultural production that will be required to meet future global demand for feed, fibre, bioenergy and food. Since this enhanced demand is largely expected to be met through yield gains on existing lands (Heffer, Prud'homme 2010), this will require greater inputs of nutrients, including P. This practise already appears to be occurring in rapidly developing countries; for example, fertiliser consumption in Asia has increased more than 10-fold in the past 40 years (Sattari et al. 2014). In addition to resource concerns, the generous use of P fertilisers (and other industrial uses of P, for example in washing powders) has created widespread economic, social and environmental problems associated with eutrophication (Dodds et al. 2009). Much of the P applied to agricultural land in the past is now stored in the soil as surplus P. This not only undermines current attempts to reverse the ecological damage and loss of aquatic biodiversity caused by eutrophication, but is also a potentially unutilised P resource, termed legacy P, that could be used to reduce applications of costly inorganic (manufactured) fertilisers, without affecting crop yields (Withers, Edwards & Foy 2001, Sattari et al. 2012, Sharpley et al. 2013).

Strategies to reduce reliance on, and make better use of, inorganic P include recovery and use of P from human and livestock waste streams, optimising the application of P fertiliser and better exploitation of existing soil P reserves (Elser, Bennett 2011, Cordell et al. 2011, Withers

et al. 2014). However, the exploitation of soil P reserves is hindered by the fact that the forms, distribution and accessibility of legacy P are complex and diverse, and often not in a form that is readily available for plant uptake. The potential store of legacy soil P is large. Withers et al. (2001) calculated that an average ca. 1000 kg ha^{-1} of surplus P is stored in UK soils in the productive arable and grassland areas. Cumulative P inputs in European cropland for the period 1965-2007 were also vastly in excess of off-take, with totals of approximately 1115 kg ha^{-1} applied, compared to off-takes of 360 kg ha^{-1} (Sattari et al. 2012). If legacy P was accounted for in nutrient planning, it has been estimated that this could reduce the requirement of inorganic fertiliser by 50% (Sattari et al. 2012).

2.2.3. Inorganic fertilisers

Along with reducing food waste, improving food distribution networks, and other factors, feeding a growing global population requires an increase in crop yields. Efficient use of fertiliser is a key component to this. Increasing global crop yields are inexorably linked with mineral fertiliser inputs, estimates place between 40 – 60% of crop yields are due to commercial fertiliser applications (Roberts 2009) (Fig. 2.2a).

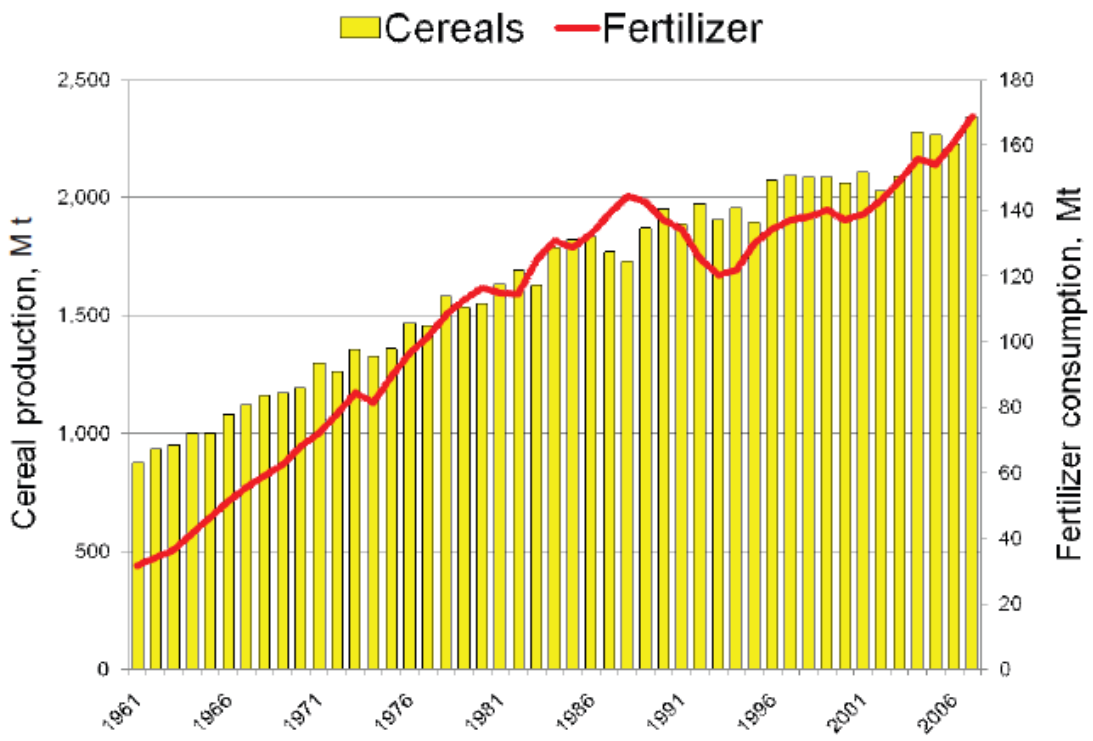

Figure 2.2*a*. World cereal production and fertilizer production, 1961- 2007 (Roberts 2009)

Within the UK however there has been a consistent reduction in the use of inorganic fertilisers (Fig 2.2b). Many factors have contributed to the reduction from increasing global prices of fertiliser to the formation of nitrate vulnerable zones, which restrict inorganic fertiliser applications. Increased yield requirments coupled with reduced fertiliser usage will require increased efficiency of applied fertilisers and that of exisiting soil nutrient reserves; within the UK therefore the use of BIs is more timely than ever.

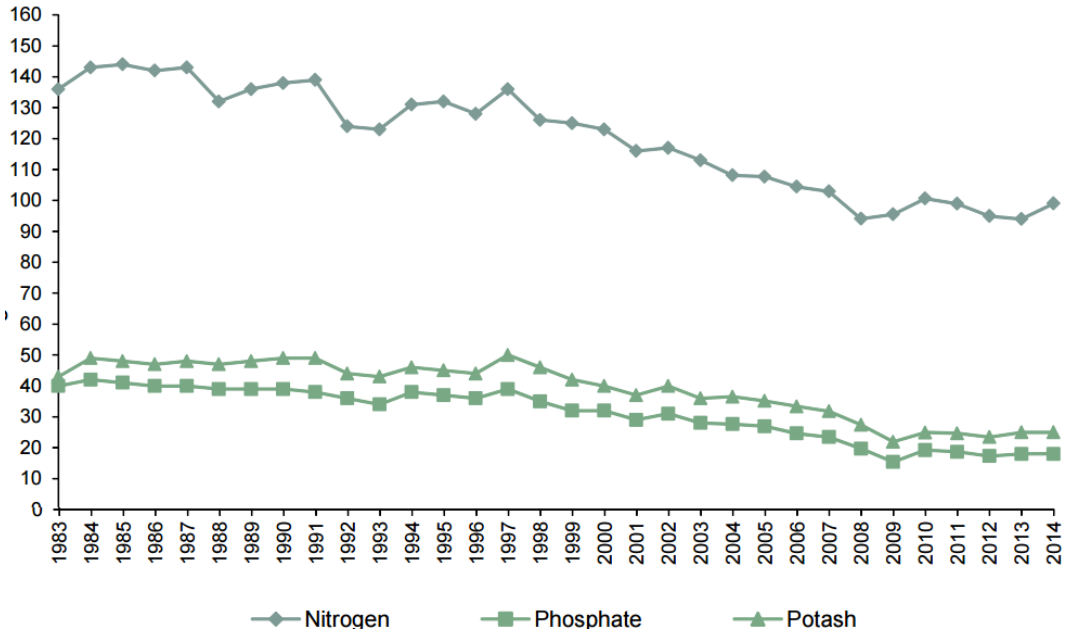

Figure 2.2*b*. UK inorganic fertiliser use, 1983- 2014 (Defra 2014b)

Mineral P fertilisers have increased total P in UK agricultural soils (Withers, Edwards & Foy 2001), for example, the utilised agricultural area in the UK is ~ 71% of the total UK area (Defra 2014a), onto which 194 kt of phosphate (P_2O_5) was applied in 2013 (with arable and grasslands receiving 28 and 9 kg ha^{-1} respectively) (Defra 2014b); resulting in an average soil P index of ~ 2. The P index of a soil is a method of quantifying the amount of plant-available P within a soil. There are several extraction methods employed to measure this plant-available soil P, largely determined by the pH of the soil being tested, for example the Olsen extraction is favoured for calcareous soils the Bray technique favoured for acidic soils (Syers, Johnston & Curtin 2008). Values of P are given as mg l^{-1} which are then classified into ranges representing P indices (Table 2.1).

Table 2.1. Classification of Olsen's soil P (mg l^{-1}) into P indices (Defra 2010)

Index	mg l^{-1}
0	0-9
1	10-15
2	16-25
3	26-45
4	46-70
5	71-100
6	101-140
7	141-200
8	201-280
9	>280

2.2.4. Fertiliser efficiency

Fertiliser efficiency depends on the capacity of a fertiliser to supply soluble P for plant uptake once applied to the soil. The availability of P is influenced by the physical, chemical and biological properties of the soil and the root characteristics of plants e.g. biochemical processes that occur at the root / soil interface (rhizosphere), effective exploration of plant roots, root exudates and associations with micro-organisms. Application of P fertiliser and some manures causes an initial sharp rise in soluble P at the point of soil contact (Allen, Mallarino 2006). Chemical equilibrium is rapidly established with soil adsorption and precipitation of lower solubility compounds. With time, soil solution P moves into less available, non-labile, pools (Yang et al. 2012). Of applied fertiliser the fraction which remains in a readily available (labile, weakly adsorbed) form is relatively small; around 13% of total soil P increase was attributed to extractable Olsen P (Johnston, Dawson 2005). However the net long term effect depends on soil chemical and mineralogical properties, P-uptake by crops, P-movement through the soil profile and soil erosion (Allen, Mallarino 2006).

2.2.5. Soil phosphorus

Phosphorus concentrations in the soil solution are inherently low (Marschner 1995). Soil P is most conveniently considered as having two origins (Withers et al. 2014): native P, which is released into the soil solution by natural weathering of the soil parent material (primary minerals; Fig. 2.3), and legacy P (Sharpley et al. 2013) the result of past applications of fertilisers and manures. As of 2000, P inputs (fertilisers and manures) across industrialised countries globally were 31 Tg $year^{-1}$, while outputs were 19 Tg $year^{-1}$, resulting in a P surplus of 12 Tg (Bouwman et al., 2011). In the UK alone, the 2013 P surplus has been calculated to be 87 Gg (Defra, 2014b), a 7.2 kg ha^{-1} surplus (Fig. 2.3). This poses an additional environmental risk from run-off or leaching (Fig. 2.3). Global soil P surpluses continue to grow and could rise to as much as 18 Tg $year^{-1}$ by 2050 (Bouwman et al., 2011). Changes to farm management could reduce P surpluses by 20%, e.g. shifting from beef to poultry, or solely arable systems to mixed arable and livestock, and improving manure management (Bouwman et al., 2011). There is also potential for BIs to reduce P inputs by exploiting the accumulating P surpluses in the soil.

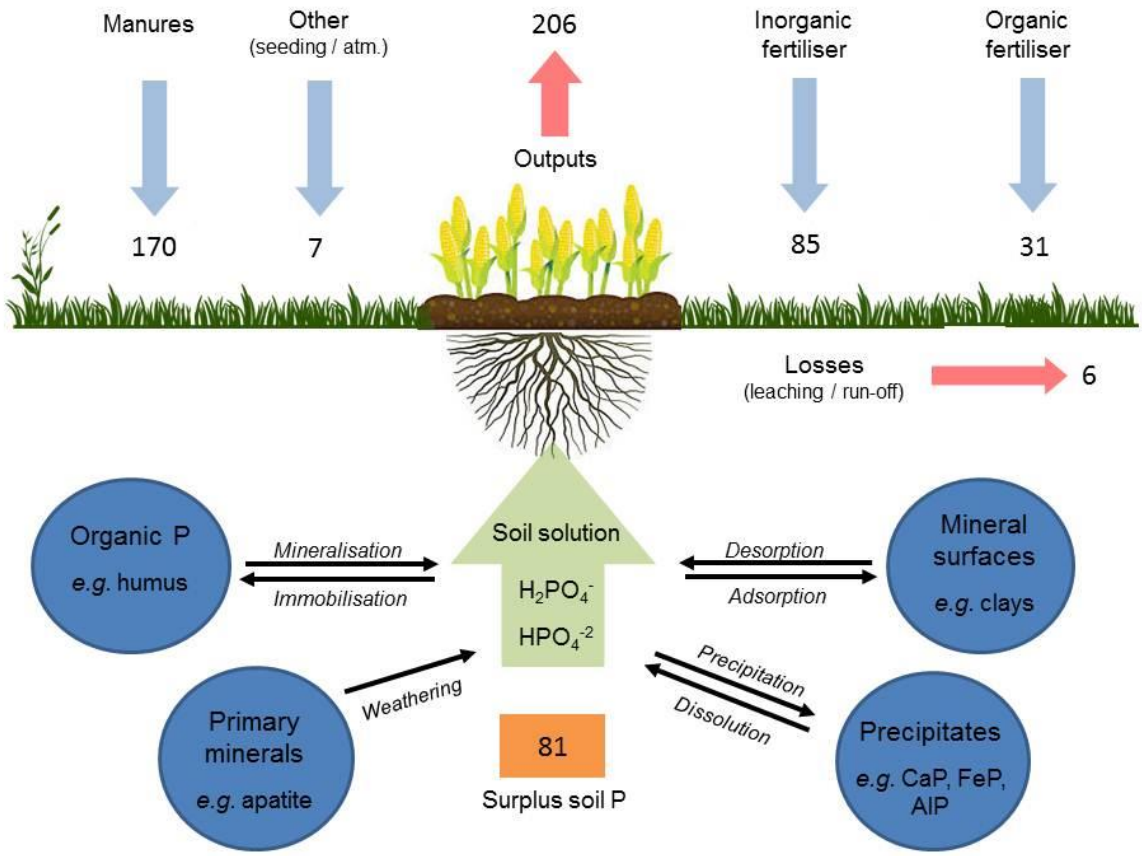

Figure 2.3: Scheme showing the various phosphorus pools and the complex web of phosphorus dynamics within soil. Blue arrows represent phosphorus inputs, red arrows represent phosphorus outputs, and the orange box represents surplus soil P (inputs – outputs). Figures represent quantities of P (Gg) cycled within UK agricultural land for 2013 (Defra, 2014b). Fertiliser inputs include both inorganic and organic fertilisers (e.g. compost and sewage sludge) but exclude livestock manure, as such internal re-cycling of P containing products (i.e. manures from dairy stock) are treated as an input. Outputs include marketed crops, fodder crops and grass (harvested and grazed) (Defra, 2014b). Runoff based on loss of 0.5 kg P ha^{-1} $year^{-1}$ (Ulen et al. 2007) on managed agricultural land area (Defra, 2014b); atm = atmospheric deposition

The release of legacy P into the soil solution depends on the form in which it is predominantly held, but it appears to be more plant-available than native P (Johnston et al. 2014). P species is largely determined by soil pH (Fig. 2.4); between a soil pH of between 4 and 10 the dominant P species are $H_2PO_4^-$ and HPO_4^{2-} (Fig. 2.4),

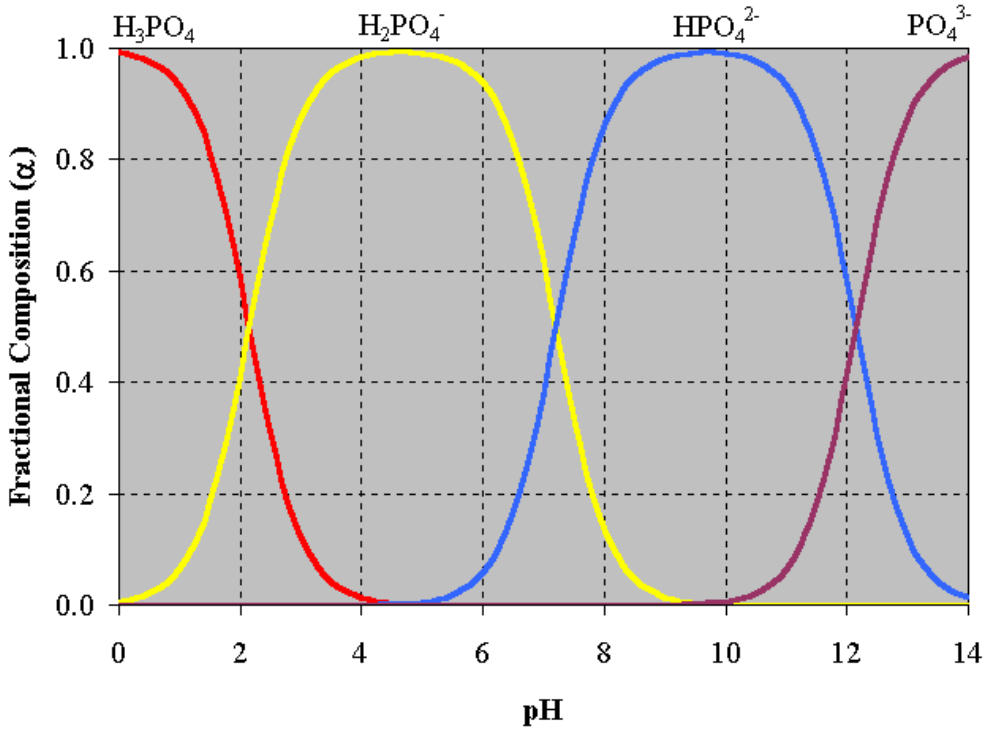

Figure 2.4. The acid dissociation constants for phosphoric acid are quite different from each other with pK_a's of 2.15, 7.2, and 12.15 because the pK_a are so different, the protons are reacted at different pH's (http://ion.chem.usu.edu/~sbialkow/Classes/3600/alpha/alpha2.html)

Plant uptake studies have shown that the inorganic mono / divalent phosphate ion, $H_2PO_4^-$ and HPO_4^{2-}, constitutes the bulk of plant P assimilation; and although there is some evidence of plant uptake of DNA (nuclease-resistant analogue of DNA) (Paungfoo-Lonhienne et al. 2010), generally the organic P forms must be mineralised; a process mediated by enzymes, chiefly phosphatase and phytase (enzymatic dissolution).

In the absence of a pool of readily-available P provided by inorganic fertilisers, plants must utilise numerous strategies to acquire soil inorganic (P_i) and organic (P_o) quickly and efficiently to ensure an adequate supply of P during the growing season (Richardson, Simpson 2011). Soil P is transformed to the plant-available phosphate ion via one of several mechanisms, dissolution/precipitation (mineral equilibria), sorption/desorption (interactions between P and mineral surfaces) and mineralization/immobilization (transformation of P_o to P_i by biological transformations) (Fig. 2.3). P_i can constitute between 35 to 70% of total soil P, whilst the P_o fraction can comprise 30 to 65%, and in soils with high (>20%) organic matter, can increase to as much as 90% (Jones, Oburger 2011). P_i both transformed and/or applied as fertilisers are subject to fixation by soil constituents. Reduced plant soluble phosphate complexes are formed when, for example, hydrous iron and aluminium oxides and aluminosilicates, a feature of acidic

soils, react with phosphate solutions to produce iron and aluminium phosphates, whereas calcareous soils promote the precipitation of calcium phosphates (Stevenson and Cole, 1999).

2.3. Bio-resources

There is much interchangeable and confusing use of terms such as bio-inoculant, bio-fertiliser and bio-amendment in the literature. We propose a hierarchical classification in which each is a separate and exclusive group under a generalised classification of 'bio-resources'. Bio-resources can be defined as any organic material applied to soil to improve soil quality, nutrient supply and plant growth.

A classification with regard to nutrient supply is shown in figure 2.5. Other mechanisms of plant growth promotion (e.g. bio-stimulation) are not shown, but would follow the same principle as laid out in figure 2.5. Bio-resources can be separated into those that primarily supply macro- and micro-nutrients for both plants and native soil biota (bio-amendments and bio-fertilisers) and those that provide a secondary nutrient supply by facilitating nutrient acquisition through stimulation or specific adaptations of the soil microbial community composition (bio-inoculants). All types of bio-resource have both primary and secondary nutrient supply functions (Fig. 2.5), but they can be usefully separated according to their form and principal mode of action:

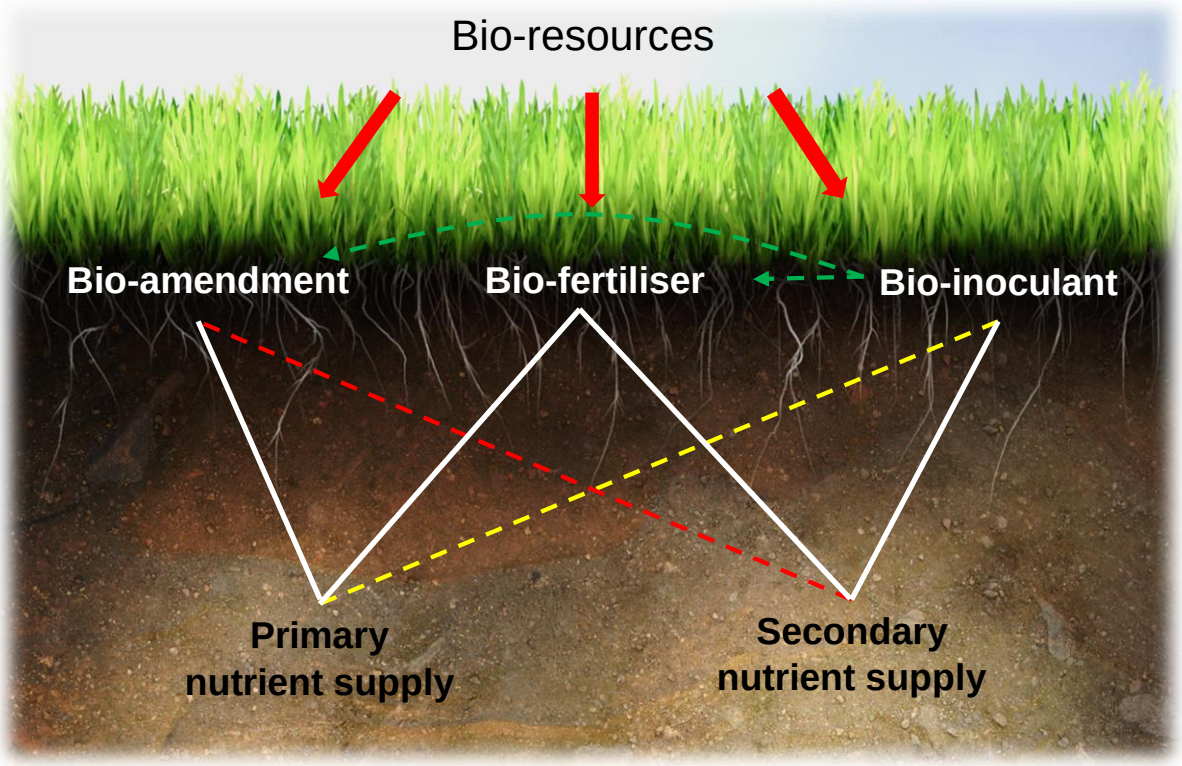

Figure 2.5. Different forms of bio-resources with respect to plant nutrient supply. **Red dotted line** = Bio-amendments may also contain and/or stimulate native micro-organisms that provide a secondary nutrient supply. **Green dotted line** = BIs added to accelerate microbial decomposition and nutrient release; **Yellow dotted line** = possible source of primary nutrient supply present within the cells of the inoculants added, made available to plants during microbial turnover

Bio-amendment – a raw organic amendment recycled directly to the soil e.g. livestock manure and green manures. These supply nutrients in inorganic form for direct uptake and in organic form for subsequent mineralisation to inorganic forms (Vessey 2003). However, they may also contain microbial populations (e.g. rhizobacteria) for secondary nutrient supply and other plant growth promotion functions (Perumal et al. 2012).

Bio-fertiliser – a product which may contain micro- / macro-plant nutrients (primary nutrient supply) or specific organic components which, directly or indirectly, stimulate microbial activity and thereby increase mobilisation of nutrients from soil (i.e. secondary nutrient supply). Examples include the use of plant enzymes and hormones, anaerobic digestates or tailored materials designed to respond to specific biological cues such as rhizosphere-controlled fertiliser (Erro et al. 2007).

Bio-inoculant – individual strains or consortia of known microbes that have potential plant growth-promoting benefits (Plant growth-promoting micro-organisms; PGPM) added directly to the soil, or as a seed coating when re-seeding. They are tailored formulations utilising current understanding of micro-organism function to create BIs for a range of soil type and cropping systems (Roesti et al. 2006, Ahmad et al. 2013). Those micro-organisms with specific attributes for mobilising RP and legacy soil P are termed phosphorus-mobilising micro-organisms (PMM).

2.3.1. Bio-inoculant composition and function

The full range of mechanisms whereby PGPM used in BIs lead to improved plant rooting, growth and crop yield are not fully understood; individual PGPM may possess multiple plant growth-promoting traits (Fig. 2.6) which influence plant growth not only directly but also indirectly via indigenous PGPM. Disentangling the contribution to plant growth of each PGPM and elucidating potentially complex interactions with other micro-organisms is clearly challenging. In addition to the primary benefit of improving nutrient supply to plants, a plethora of other effects may be significant, for example the production of metabolites (phytohormones, antimicrobials, antibiotics) which alter root development or inhibit other microbes (e.g. pathogens). The constituent PGPM used in BIs can be categorised into two main groups, bacteria and fungi (Fig. 2.6). These groups can be further categorised into intracellular and inter / extracellular for bacteria (Gray, Smith 2005); and Root-associated fungi (RAF), Ecto-mycorrhizas (EcM) and Arbuscular-mycorrhizas (AM) for fungi.

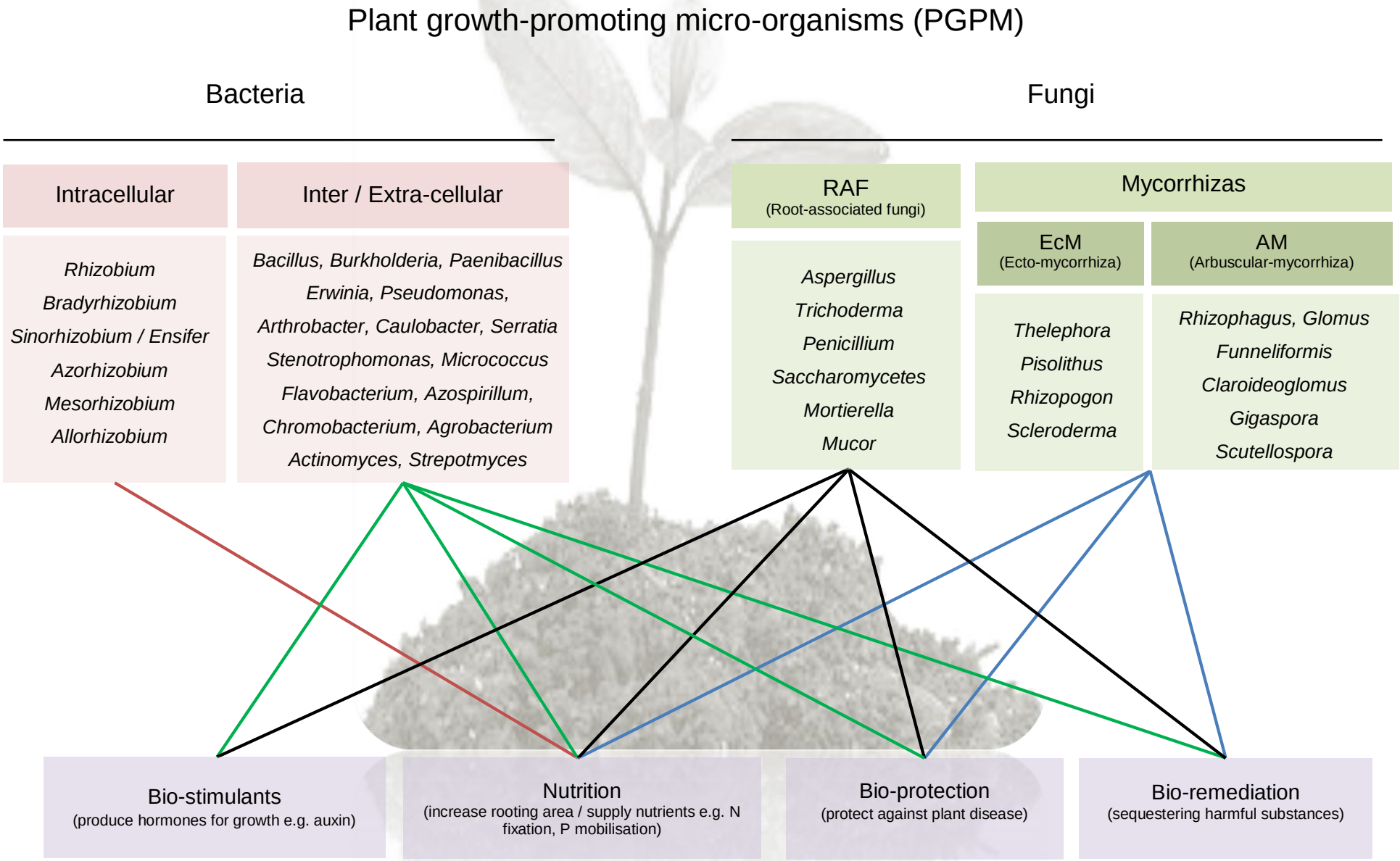

Figure 2.6. Main plant growth-promoting groups (genera in italics) used in commercial bio-inoculants and the various mechanisms each employ to promote plant growth

2.3.2. Bacteria

Mechanisms of plant growth promotion include hormone production, improved plant nutrition (mainly N and P), reduction of plant ethylene levels and induced systemic disease resistance. They can indirectly increase growth through bio-control mechanisms of reducing plant disease or stimulating other microbial symbioses e.g. mycorrhizas (for a detailed review see Antoun, Prevost (2005) and Martínez-Viveros et al. (2010)). Bacteria can be split into two groups with respect to the mechanism of plant association (Fig. 2.6). Intracellular bacteria are those which reside within plant cells, producing nodules localised inside specialised structures; mostly N-fixing rhizobia. Inter / extra-cellular bacteria are those which reside outside the plant cell and do not produce nodules but may still reside within the plant tissue (intercellular), in apoplastic spaces (endophyte), or on the rhizoplane (extracellular) (Compant, Clément & Sessitsch 2010), or even in the phyllosphere (Compant et al. 2008). Inter / Extra-cellular bacteria promote plant growth through the production of a variety of stimulating compounds e.g. hormones, antibiotics and enzymes (Gray, Smith 2005).

There are many bacterial commercial products each claiming enhanced plant disease suppression (bio-protectant), nutrient acquisition improvement and/or phytohormone production (bio-stimulants). P improvement mechanisms mediated by bacteria include the production of phosphatases (both alkali and acid), siderophores (Franco-Correa et al. 2010) and lowering of pH through acid secretion (Illmer, Schinner 1992). The P-mobilising potential of Actinobacteria has seen them exploited as BIs (Pragya, Yasmin & Anshula 2012) e.g. Micro 108, a soluble BI containing *Streptomyces lydicus* (WYEC 108) (Kowalski 2010); the company citing ligand exchange as the main mechanism of increased nutrient availability through the production of siderophores. However, the response of plants to soil inoculation in the field is difficult to predict (Antoun, Prevost 2005). The native bacterial populations within the soil may affect the performance of the inoculant, whilst abiotic factors (e.g. soil pH, water content and temperature) will impact on both native and introduced bacterial species (Antoun, Prevost 2005).

2.3.3. Root-associated fungi (RAF)

As with bacteria, RAF may reside within the rhizosphere, on the rhizoplane, and in many cases within root tissues (endophyte). RAF confer plant beneficial effects through several mechanisms, for example through inducing resistance to disease and tolerance to abiotic stresses (Waller et al. 2005, Rawat, Tewari 2011). For instance, *Trichoderma* spp. synthesise

auxins which stimulate lateral plant root development (Benitez et al., 2004; Contreras-Cornejo et al., 2009) or modify host synthesis of nitric oxide under conditions of pathogen attack (Gupta et al., 2000). Other functions have been categorised as bio-control and bio-remediation (Rodriguez et al. 2009) (Fig. 2.6). RAF are able to both solubilise P_i and mineralise P_o, e.g. *Aspergillus* and *Penicillium* spp. inhabiting the rhizosphere can secrete organic acids which mobilise P_i from rock phosphate, as well as phosphatase enzymes, allowing hydrolysis of P_o (Bolan 1991, Vassilev, Fenice & Federici 1996, Barrow, Osuna 2002).

2.3.4. Mycorrhizas

The term 'mycorrhiza' is derived from the Greek *myco-* (fungus) and *rhiza* (root). Mycorrhizas, whilst are associated with plant roots, differentiate themselves from RAF by way of an extensive (extraradical) network of hyphae in the soil which acts as an extension of the plant's roots system. Efficient exchange of nutrients (sucrose to the fungus and N / P to the plant) is mediated via specialised structures within the roots (e.g. intra-cellular arbuscules in AM fungi). The basis of these mutualisms is the ability of fungi to form fine hyphae (with more favourable surface area to volume ratio for nutrient uptake) and to secrete enzymes / organic acids to mobilise nutrients. Some 90% of plants form mycorrhizal symbioses (Smith, Read 2008); 83% of dicotyledonous plants and 79% of monocotyledons (Peterson, Massicotte & Melville 2004). They are categorised into seven main groups: arbuscular (AM), ecto- (EcM), ectendo-, arbutoid, ericoid, monotropoid, and orchid mycorrhiza (Smith, Read 2008). AM and EcM are the most widespread and ecologically important types of mycorrhiza and the only ones commercially exploited in agriculture / forestry (Fig. 2.6).

AM fungi belonging to the phylum Glomeromycota are the most widely used in agriculture, notably *Rhizophagus* (formerly *Glomus*) *intraradices* and *Funneliformis* (formerly *Glomus*) *mosseae* (Krüger et al. 2012); both of which have been shown to increase P uptake in diverse crop plants (Barea, Bonis & Olivares 1983, Douds et al. 2007, Antunes et al. 2009, Cozzolino, Di Meo & Piccolo 2013, Williams, Ridgway & Norton 2013). However, AM fungi are obligate symbionts which can be grown only in the presence of host plants. Thus, BIs comprising them contain preparations of spores propagated in pot cultures mixed with an inert carrier (Gentili, Jumpponen 2006). The use of EcM (phylum Basidiomycota) as BIs is more restricted, since they only infect temperate and boreal forest trees. However, in plantation forestry, both the establishment success and early growth rate of saplings inoculated in nurseries with EcM is known to be enhanced (Dalong et al. 2011, Oliveira, Franco & Castro 2012).

Recent advances have also led to the discovery of new groups of mycorrhizal fungi, for instance *Piriformospora indica* (phylum Basidiomycota; order Sebacinales) which appears to have considerable potential as a BI. For example, when *P. indica* was co-inoculated with a plant growth-promoting bacteria (pseudomonads), N and P levels in the host (*Vigna mungo*) almost doubled (Kumar et al. 2012). Additionally *P. indica* is not an obligate endosymbiont, so can be readily cultured axenically (Varma et al. 1999) and it can also form mutualistic interactions with groups of crop plants (e.g. *Brassicaceae*) which were previously considered to be non-mycorrhizal (Camehl et al. 2011).

Outside the root, the fungal mycelium of mycorrhizal fungi forms an extensive network within the soil and leaf litter. The mycelial network extends the plant root rhizosphere, increasing nutrient absorption potential. Mycorrhizas are particularly important for increasing the uptake of slowly diffusing ions such as PO_4^{3-}, and immobile nutrients such as Zn and Cu (Clark, Zeto & Zobel, 1999). The mycelial network is able to solubilise and mineralise P through the production of organic acids and enzymes, respectively (Marschner 1995, Koide, Kabir 2000), and has been shown to improve productivity in soils of low fertility, especially P (Richardson, Simpson 2011, Smith et al. 2011). Colonisation of roots by mycorrhizal fungi has been shown to be negatively correlated with P fertilisation (Treseder 2004).

The extraradical mycelia of mycorrhizal fungi also supports a microbial community (Andrade, Linderman & Bethlenfalvay 1998), in much the same way as the plant rhizosphere does, termed 'myco-rhizosphere' (Rambelli 1973). This hyphal-based microbial community may also mineralise and solubilise recalcitrant nutrient complexes which are then transported to the plant via the mycelial network (Toljander et al. 2007). Seedling tolerance to various stresses e.g. drought, high temperature, toxic heavy metals and pH extremes is also increased (Gupta, Satyanarayana & Garg 2000).

2.3.5. Phosphate-mobilising micro-organisms (PMM)

Organisms that specifically mobilise native and legacy soil P and any insoluble source of P added (e.g. finely ground RP) are generally referred to as phosphate-solubilising micro-organisms. This terminology however is slightly misleading as phosphate-solubilising micro-organisms also mobilise organic P through enzymatic hydrolysis (mineralisation) and facilitate the translocation of phosphate (Jones, Oburger 2011). A more accurate terminology would therefore be phosphate-mobilising micro-organisms (PMM) (Fig. 2.7).

Microbial solubilisation of P is widely thought of as the 'organic acid theory', in which the two mechanisms of P acquisition involve lowering of pH (directly dissolving mineral P, by proton extrusion) and / or by the release of organic acid anions that exchange for P on soil adsorption sites (ligand exchange) (Oburger, Jones & Wenzel 2011, Zhang et al. 2011). Organic acid anions most commonly released by PMM include succinic, citric, gluconic, α-ketogluconic and oxalic (Chen et al. 2006). Whilst PMM may directly mobilise both P_i and P_o, indirect mobilisation of P also occurs through:

i) CO_2 released from microbial respiration and which dissolves in soil water to form carbonic acid, solubilises P through reduced myco-rhizosphere pH (Marschner 1995)

ii) Redox activity of micro-organisms and/or exudates (secondary organic metabolites, siderophores, enzymes, phenols, amino acids, sugars and organic acid anions reduce metals with variable oxidation states (bound to phosphate) to a lower oxidation state, resulting in more soluble phosphate (Kim, Jordan & McDonald 1998))

iii) Nitrogen assimilation, where protons are exuded following microbial assimilation of ammonium (NH_4^+). Excreted H^+ accompanying the decrease in pH act as a solvent agent for P solubilisation (Illmer, Schinner 1992)

iv) Sink theory – P-solubilizing organisms are able to remove and assimilate P from the media and thus stimulate the indirect dissolution of Ca-P compounds to re-establish solution P equilibrium (Halvorson, Keynan & Kornberg 1990)

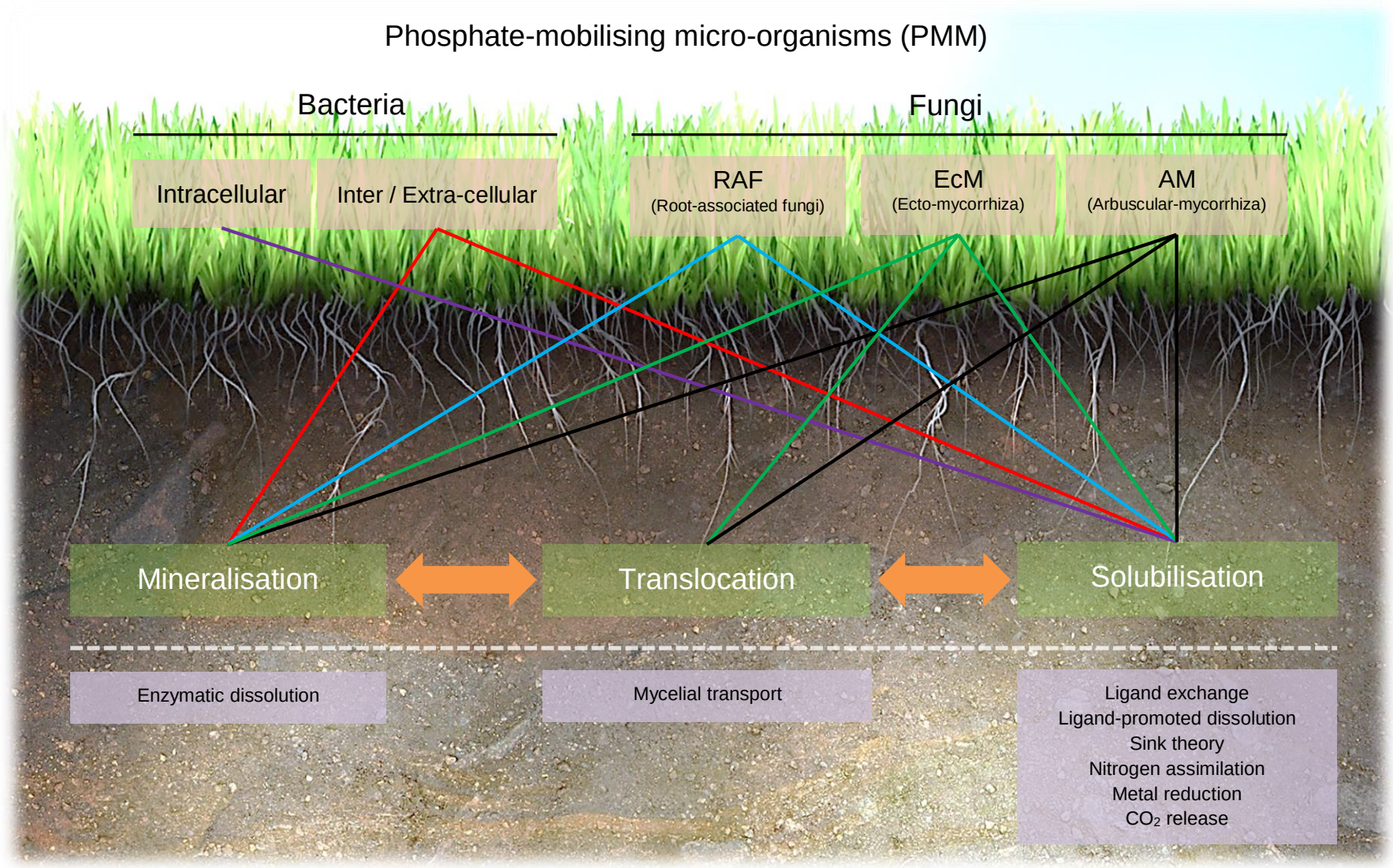

Figure 2.7. The three main processes of phosphate mobilisation and mechanisms used by phosphate-mobilising micro-organisms. Orange arrows represent potential P mobilisation by organisms associated with fungal hyphae (mycor-rhizosphere)

2.4. Markets and quality control

The majority of BIs used in 2012 were mostly rhizobium (nitrogen fixing bacteria), constituting 79% of the global demand. Phosphate-mobilising BIs were ~15%, with other BIs, such as mycorrhizal products, making up ~7% (Transparency Market Research 2014). While the market is dominated by N-fixing products, it is expected that P-mobilising products (incl. mycorrhizal) will see increased demand. To date, there appears to be about twelve producers of mycorrhizal inocula in the EU, with producers in the UK, Czech Republic, Germany, Switzerland, Spain and France, and more than 20 others worldwide (the majority residing in the USA) (Vostaka, Albrechtova & Patten 2008).

Regulation and quality control of BIs is hindered by the natural diversity in bacterial and fungal species and variability in their functions, resulting in a lack of a general standard for bacterial and fungal performance and viability (Brahmaprakash, Kumar Sahu 2012). There is much scope for BIs to vary in their effectiveness/activity, for instance if not stored under conditions that maintain microbial viability or if different culture batches vary in their viability or vigour. Many nations only have voluntary standards for rhizobial products (Bashan 1998) e.g. Australian Inoculants Research and Control Service, maintained by manufacturers of *Rhizobium* inoculants (Kennedy, Choudhury 2002). India however has seen regulatory standards developed for *Azotobacter*, *Azospirillum*, PMM and mycorrhizal inoculants (Yadav 2009). The EU has yet to implement such regulatory standards, and quality control is left to market forces. Current EU fertiliser regulation covers inorganic fertilisers (EC No 2003/2003); however there is scope to extend this regulation to cover organic fertilisers and BIs. The formation of standards may pave the way to eliminate inferior products from the market place that undermine confidence in the sector (Olsen, Rice & Collins 1995). The lack of regulation, and concerns that this may raise within consumers, may have been somewhat addressed through the formation of voluntary guilds e.g. The Federation of European Mycorrhizal Fungi inoculum Producers, which has seen 40 companies sign up. Similarly, in the US, the International Mycorrhizal Manufacturers Association and the International Mycorrhizal Society were established to provide standards for global manufacturers of mycorrhizal products and improve confidence in the sector.

2.5. Bio-inoculant performance

2.5.1. Evidence of efficacy

The success of commercial BIs should be reflected in an economic gain, either through improved yields or reduced inorganic fertiliser applications, or both. They should not be viewed as a replacement for inorganic fertilisers, but as a potential component of an integrated nutrient management strategy that enhances soil nutrient acquisition (e.g. from native and legacy P). There is a plethora of products on the market, all of which claim to consist of PGPM, either as single strains or consortia. There is a shortage of peer-reviewed publications that report on studies using commercially available BI products, but a summary of recent research with respect to grass (mainly *Lolium perenne*) and arable crops (mainly *Zea mays*) is given in Table 2.2. The results are varied, inconsistent and contradictory, which may discourage companies to allow their products to undergo a rigorous scientific examination. Some researchers do not reveal the products used, for reasons of intellectual proprietary or protection of commercial marketability (Tarbell, Koske 2007). Of the literature that is available, there is no consensus on the efficacy of BI products; of the few products which are demonstrated to have a clear (non-substrate induced) plant growth effect, the mechanism of enhancement has not been fully elucidated. A survey of different products by the authors revealed that a number (primarily bacterial) have a very short shelf-life, whereas for other products, 'use-by' dates were completely absent. The stabilisation of the products with time therefore seems to be highly variable. This lack of consistency in performance between different batches of the same product has been highlighted previously (Wiseman, Colvin & Wells 2009). Furthermore, the distinct lack of consistency in application rates and methods for the different products is of concern (Table 2.2).

There is a distinct lack of robust field-based testing of commercial BIs; the majority of studies focusing on laboratory-based pot trials (Table 2.2). It has been suggested that experimental design is often not sufficiently rigorous to provide the statistically-validated evidence to support a direct beneficial PGPM effect (Jones, Oburger 2011). The efficacy of any fungal or bacterial strain utilised within inoculants is also subject to numerous soil, crop and environmental factors, from crop species compatibility, size and effectiveness of indigenous microbial populations, soil fertility and management (Adholeya, Tiwari & Singh 2005), to priority effects, in which initial populations of species determine final community composition

(Mummey, Antunes & Rillig 2009, Verbruggen et al. 2013). Collectively, these affect the soil microbial dynamics, functional processes and hence performance of commercial BIs.

Table 2.2. Peer-reviewed publications that have utilised commercially available BI products. Studies highlighted in red in the results column indicate that the BIs did not improve growth / biological parameters of the respective experiment; studies in green gave a positive result. Species names are as reported within each paper (NB: many AM fungi formerly known as *Glomus* spp. have recently been renamed). Shoot P is a measure of the amount of phosphorous contained within the aboveground biomass; DMY (dry matter yield)

Product	PGPM	Crop	Field / Lab	Result	Application rate	Sterilised controls	Ref.
Mycormax JH Biotech Inc. Ventura, US	2 × *Glomus* 5 × Ectomycorrhizal	*Zea mays*	Lab (sterilised soil)	<5% root colonisation. Increased DMY	1.2 g l^{-1} soil	No	Wiseman, Colvin & Wells 2009
BEI Bio Organics Santa Maria, US	6 × *Glomus* 1 × *Gigasporaceae* 1 × *Paraglomus*		Lab (sterilised soil/sand)		1.8 g l^{-1} soil		
AgBio Endos AgBio Inc. Westminster, US	6 × *Glomus* 1 × *Gigasporaceae*				3.0 g l^{-1} soil		
AM 120 Reforestation Technologies Int., Salinas, US	3 × *Glomus*			<5% root colonisation. No increase in DMY	3.0 g l^{-1} soil		
BioGrow Endo Mycorrhizal applications Inc., Grants, US	3 × *Glomus* 1 × *Trichoderma*			<5% root colonisation. Increased DMY	3.0 g l^{-1} soil		
Die Hard Endo starter Horticultural alliance Inc., Sarasota, US	6 × *Glomus* 1 × *Gigasporaceae* 9 × Ectomycorrhizal 1 × *Trichoderma*				1.2 g l^{-1} soil		
Mycor Tree root dip Plant Healthcare Inc.	5 × ecto/endomychorrizae			<5% root colonisation. No increase in DMY	Mix with water (not specified)		
Root dip universal Tree pro, West Lafayette, IN	AM spores (not specified) Beneficial bacteria						

Mazospirflo-2 Soilgro Ltd., South Africa	*Azospirillum brasilense*	*Zea mays*	Lab (field soil)	No increase in DMY or shoot P	200 ml 25 kg^{-1} seed	No	Laditi et al. 2012
Eco-T Plant Health Products (Pty) Ltd. South Africa	*Trichoderma harzianum*, Strain Rifai KRL AG2	Soybean		No significant increase in nodule number/size, shoot P or DMY	1 g kg^{-1} seed		
PHC-Biopack Plant Health Products (Pty) Ltd. South Africa	*Bacillus* *Paenibacillus azotofixans*			Increased nodule mass. No increase in shoot P or DMY	2 teaspoon 4.55 l^{-1} water		
EM - Bioaab Nature Farming Research and Development Foundation, Faisalabad, Pakistan	*Lactobacillus plantarum* *Lactobacillus casei* *Streptococcus lactis* *Rhodopseudomonas palustris* *Rhodobacter sphaeroides* *Saccharomyces cerevisiae* *Candida utilis* *Streptomyces albus* *Streptomyces griseus* *Aspergillus oryzae* *Mucor hiemalis*	*Vigna mungo*	Lab (sandy loam soil)	48% increase in grain yield in combination with NPK fertiliser	500 ml EM dilution (1:1000)	No	Javaid 2009
EM Bionova Hygiene GmbH, Stans, Switzerland		Rotational crops (potatoes, winter barley, alfalfa, winter wheat)	Field (medium utric Regosol)	Did not improve yields or soil quality	110 l ha^{-1} (applied as a dilution of EM-1 (5% v/v))	Yes	Mayer et al. 2010
EM Punto EM, Sanremo, Italy		*Lolium perenne*	Lab (hydroponics)	35% increase in root length. Increased microbial diversity at root level. No significant increase in shoot DMY	Not specified	No	Baffoni et al. 2012

EM-1 Not specified	*Lactobacillus plantarum* *Lactobacillus casei* *Streptococcus lactis* *Rhodopseudomonas palustris* *Rhodobacter sphaeroides* *Saccharomyces cerevisiae* *Candida utilis* *Streptomyces albus* *Streptomyces griseus* *Aspergillus oryzae* *Mucor hiemalis*	*Lolium perenne*	Lab (loamy calcareous chernozem soil)	Increased soil urease and dehydrogenase when combined with NPK	15 ml kg^{-1} growth media	No	Jakab et al. 2011
EM-1 EMIKO GmbH, Euskirchen-Kirchheim			Lab (field soil)	Marginal effects on C_{org}, N_{total}, N_{min}, microbial activity in soil. No increase in DMY	10 l ha^{-1}	Yes	Schenck zu Schweinsberg-Mickan, Müller 2009
EM Not specified		*Zea mays*	Field (sandy loam)	When used in combination with other microbial inoculants reduced inorganic inputs by 50%. Also increased populations of rhizospheric azotobacter	2.5 l ha^{-1}	No	Jilani et al. 2007
Biopower Not specified	*Azotobacter*, *Azospirillum*, *Azoarcus*, *Zoogloea*			When used in combination with other microbial inoculants reduced inorganic inputs by 50%	2.5 kg ha^{-1}		
Biostimulator Agrinova GmbH	*Bacillus subtilis* *Ascophyllum nodosum*	*Lolium perenne*	Lab (field soil)	No effects on mineral N in soil. Suppressive effects on microbial-biomass content and activity. No increase in DMY	3 kg ha^{-1}	Yes	Schenck zu Schweinsberg-Mickan, Müller 2009
Bactofil-B Agrinova GmbH	*Azospirillum brasilense*, *Azospirillum lipoferum*, *Azotobacter vinelandii*, *Bacillus megaterium*, *Bacillus polymyxa*, *Bacillus circulans*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Streptomyces albus*				10 l ha^{-1}		
Bactofil-A Not specified			Lab (chernozem soil)	Increased soil urease and dehydrogenase when combined with straw	20 ml kg^{-1} growth media	No	Jakab et al. 2011
Bactofil-A Agrinova GmbH			Lab (field soil)	No effects on mineral N in soil. Suppressive effects on microbial-biomass content and activity. No increase in DMY	10 l ha^{-1}	Yes	Schenck zu Schweinsberg-Mickan, Müller 2009

Microbion UNC not specified	Bacterial (not specified)	*Lolium perenne*	Lab (chernozem soil)	Increased soil urease and dehydrogenase	10 mg kg^{-1} growth media	No	Jakab et al. 2011
		Armoracia macrocarpa	Field (chernozem soil)	Increased N content. No increase in plant P	2 kg ha^{-1}		Kovacs, Sipos 2009
MYKE PRO SG2 Premier Tech Biotechnologies, Canada	*Glomus intraradices*	*Zea mays*	Lab (mix of sterilised and field soil)	The inoculant significantly improved the P content of the host but only in presence of the resident AM fungal community	7.5 kg ha^{-1}	Yes	Antunes et al. 2009
		Solanum tuberosum L. cv. Superior	Field (Berks shaley loam)	Increased total fresh weight yield in a high P soil (375 mg kg^{-1})	15 cm^3 $plant^{-1}$	No	Douds et al. 2007
Aegis® Italpollina, Rivoli Veronese, Italy		*Zea mays*	Field	Similar plant growth to NPK treatments	25 kg ha^{-1}		Cozzolino, Di Meo & Piccolo 2013
Earth Roots Not specified	*Various MF*	*Zea mays*	Lab (Sterilised nursery mix, bark sawdust clay and sand)	Successfully colonised roots 2 weeks after planting	1 teaspoon pot^{-1}	No	Corkidi et al. 2004
MycoApply Endo Not specified	*Glomus intraradices*				10 g pot^{-1}		
VAM 80 Not specified				Successfully colonised roots 6 weeks after planting	1 teaspoon pot^{-1}		
Ascend PB Not specified					1 g pot^{-1}		
NTC Not specified				Did not promote mycorrhizal colonisation	30.5 ml pot^{-1}		

2.6. Effects of product types, carrier media and persistence

Known plant-beneficial organisms can be isolated and sub-cultured into pure cultures, and subsequently used in various formulations of BIs, including wettable powders, granules and bacterial liquid suspensions. Whilst spores persist longer within the soil environment, they are slow to colonise host plants compared to fragments, therefore inoculants generally consist of both (Marin 2006).

Ensuring consistency in product type and formulation appears challenging to industry, even between supposedly similar products (Table 2.2). The choice of growth media has also shown to be a major factor affecting inoculant success. Using ten commercially available products Corkidi et al. (2004) found colonisation rates on *Zea mays* ranging from 0 to 50%, with no significant effect on overall plant growth. Similarly, Tarbell and Koske (2007) found that of eight AM fungal products tested, only three successfully colonised roots of *Zea mays*, with colonisation rates of between 0.4 and 8%. The authors also found that one of the inoculants tested, which failed to produce mycorrhizas, contained the root pathogen *Olpidium brassicae*.

None of the laboratory-based trials were conducted under gnotobiotic conditions. Some trials used sterilised seeds (e.g. Javaid, 2009; Wiseman, Colvin & Wells 2009; Antunes et al. 2012; Laditi et al. 2012), and autoclaved growth media, but the temperatures used may have been inadequate for complete bacterial sterilisation (Wiseman, Colvin & Wells 2009). Several studies did not use sterilised (i.e. heat-killed inoculant) controls, thus preventing the assessment of any potential plant growth effects from the bio-inoculum carrier media (Table 2.2). This is of importance given that the substrate carriers of some inoculants have been shown to induce greater plant growth over the microbes they contain (Corkidi et al. 2004, Schenck zu Schweinsberg-Mickan, Müller 2009, Wiseman, Colvin & Wells 2009, Hale 2012). The choice of carrier substrate of the inoculant is important as the substrate should provide a stable environment for the microbial fractions, prolong product shelf-life and is also the vector for dispersal or dissolution within soil, post-application (Malusá, Sas-Paszt & Ciesielska 2012). Carriers can consist of soil materials (peat), clays (vermiculite and zeolite), organic materials (composts, coal) or inert materials e.g. perlite (Smith 1992, Malusá, Sas-Paszt & Ciesielska 2012). Liquid inoculants can be based on broth cultures, mineral or organic oils, or on oil-in-water suspensions (Malusá, Sas-Paszt & Ciesielska 2012).

2.7. Multiple benefits, microbial interactions and adaptations

BI design frequently utilises many genera of micro-organisms, offering functional redundancy and / or added plant benefits other than increased P supply. The majority of the non-commercial literature concerning the beneficial effects of PGPM focuses on individual microbial species or strains (Vessey 2003, Aseri et al. 2008, Prasad et al. 2012). Single species PGPM can have beneficial effects (Roesti et al. 2006, Jansa, Smith & Smith 2008), but mixed inocula have been shown to be more flexible and productive within variable abiotic and biotic environments (Malusá, Sas-Paszt & Ciesielska 2012). It would be prudent for a symbiosis to favour fitter AM in acquiring P. However it has been shown that a diverse community of fungi and associated bacteria are able to supply other macro- / micro-nutrients (Hart, Forsythe 2012), and confer additive benefits to host plants such as improved pathogen resistance (Oehl et al. 2001, Maherali, Klironomos 2007, Sikes, Powell & Rillig 2010). For instance, organic acid production and phosphate solubilisation was found to be greater in co-inoculated cultures of *Aspergillus niger* and *Burkholderia cepacia* than as single inoculants (Braz, Nahas 2012).

Conversely, dual inoculation has often been shown to have no effect, or even a negative effect on plant growth. For instance, positive growth responses have been reversed by dual inoculation, even though colonisation rates of the multi-inoculants remained the same (Dodd, Ruiz-Lozano 2012). *Bacillus subtilis* and *Azospirillum brasilense* did not improve plant growth compared to singly inoculated treatments (Felici et al. 2008). Microbial production of the plant growth hormone, gibberellin, by individual *Bacillus* sp., was reduced when dual inoculated (Gutierrez-Manero et al. 2001). *Bacillus* spp. have been shown to produce a broad spectrum of antimicrobial compounds which act as bio-pesticides, particularly active against gram-positive bacteria and fungi (Földes et al. 2000). *Rhizophagus irregularis* and *Pseudomonas fluorescens* increased plant dry weight of wheat (*Triticum aestivum*) infected with the pathogen *Microdochium nivale* but the plant growth-promoting bacteria, *Paenibacillus brasiliensis*, inhibited this positive effect; an interesting example of the negative interactions of well-known PGPM (Jaederlund et al. 2008).

Several mechanisms may mediate microbial antagonism, including growth inhibition by diffusible antibiotics and volatile organic compounds, toxins and bio-surfactants (Berg 2009), competition for colonisation sites / nutrients / minerals, and pathogenicity factors reduced to parasitism (Berg 2009). *Glomus* spp., used in most BIs (Table 2.2) have been shown to have

many PGPM antagonists e.g. *Aspergillus niger*, *Fusarium solani*, S*treptomyces* spp., *Trichoderma harzianum* and *T. koningii* (Edwards, Young & Fitter 1998), *Scutellospora* spp. (Jeong, Lee & Eom 2006). *Trichoderma* is a known myco-fungicide (as well as a PMM) and could potentially inhibit AM within mixed formulations ((Kaewchai, Soytong & Hyde 2009) for full antibiosis review of *Trichoderma*). Due to commercial sensitivity in disclosing blend formulations, investigating such effects is difficult with commercially available BIs; with authors not specifying strains and instead using generic terms such as 'Vesicular Arbuscular Mycorrhizas', 'Phosphate Solubilising Bacteria', and '*Azospirillum* spp.' (free-living N-fixing bacteria) (Das et al. 2007, Uyanoz 2007).

For the conifer *Podocarpus cunninghamii*, the presence of indigenous AM species (*Acaulospora laevis*) was shown to increase plant growth rates and tissue N and P, compared with non-indigenous *Glomus* spp., which exhibited a negative effect (Williams, Ridgway & Norton 2013). Similarly, fungi isolated from an acidic soil, dominated by insoluble Al and Fe phosphates, were unable to solubilise $CaHPO_4$ (Khan et al. 2009). Native AM are acclimatised (Lambert, Cole & Baker 1980) such that the plasticity to edaphic stresses is maintained when the AM is cultured within a similar environment (Enkhtuya, Rydlová & Vosátka 2000). Maherali and Klironomos (2007) found that plant biomass in communities derived from native field soil was similar to that in the most productive experimentally-assembled fungal communities. This would have a bearing on inoculum formulations using strains cultured from un-stressed environments e.g. indigenous AM (*Funneliformis mosseae*) of a mine waste soil increased transfer of arsenic from root to shoot in *Plantago lanceolata*, whilst non-indigenous *F. mosseae* restricted plant absorption (Enkhtuya, Rydlová & Vosátka 2000). Significant plant / AM co-adaptation to local nutrient conditions has been found (Antunes et al. 2012, Johnson et al. 2010). Plant growth-promoting bacteria (*P. fluorescens*) and AM increased yields in a low-nutrient input wheat crop, however PGPM isolated from the rhizosphere of the wheat plants failed to emulate the same yield gains in rice and black gram (Mäder et al. 2011).

Once established successfully, introduced AM have been shown to decrease the species richness of indigenous AM fungal communities in host roots (Koch et al. 2011). Pre-inoculation of seedlings (*Leucanthemum vulgare*) with *Glomus* spp. reduced root AM diversity within an AM-rich soil, whereas pre-inoculation with *Gigaspora* did not (Mummey, Antunes & Rillig 2009). Differing colonisation strategies between AM species may explain this difference, since the biomass of *Gigasporaceae* is mostly outside the plant roots, thus reducing

competition with indigenous AM within the rhizosphere (Mummey, Antunes & Rillig 2009). Priority effects could also be a confounding factor, in that pre-colonised roots have been shown to exhibit limited enhancement of total root colonisation by added inoculum (Wiseman, Colvin & Wells 2009). It has been shown that AM do alter mycorrhizal colonisation in the presence of other AM (Pearson, Abbott & Jasper 1993), the authors suggesting the competition between the fungi is mediated by the host plant, possibly through carbon supply.

2.7.1. Host plant specificity

Mycorrhizal growth dependency of host species can have a bearing on the success of AM colonisation (Hart, Forsythe 2012). The importance of plants within BI design and testing is highlighted by the contradictory results obtained when using differing plant species. For example, maize (Wiseman, Colvin & Wells 2009) and grapevines showed positive and negative responses respectively to a mycorrhizal inoculant Mycormax© (Baumgartner 2002), a mycorrhizal inoculant which contains two AM and five EcM fungal species.

Hart and Forsythe (2012) were able to show how host plant characteristics and soil nutrient status affect plant responsiveness to AM. Foliar nutrient levels (N, Ca, P, Mg, K, Na, Fe, Mn, Cu, Zn, and B) in the highly mycorrhizal-dependent leek (*Allium* spp.) were reduced following AM inoculation when plants were grown in a low-nutrient environment (possibly due to sequestration of nutrients by the AM). In contrast, *Plantago* spp. with low mycorrhizal dependency responded positively to AM inoculation. However, in both cases, the growth responses were reversed when soil P levels were increased, possibly indicating the parasitic nature of AM, which is dependent on the plant species mycorrhizal growth dependency and soil test P (Hart, Forsythe 2012).

2.7.2. Soils and their management

Soil type has been shown to be a major factor determining microbial community structure, as well as plant growth and rhizosphere nutrient dynamics (Oehl et al. 2010, Marschner, Crowley & Rengel 2011, Wagg et al. 2011, Yousefi et al. 2011). Corkidi et al. (2004) found significant interactive effects of a commercial (unidentified) mycorrhizal inoculant and potting medium on both shoot height and dry mass of *Zea mays*. Bashan et al. (1995), using 23 different soil types, were able to show how soil type affected the persistence of *Azospirillium brasilense* in root-free media. The authors highlighted abiotic and biotic soil factors had both positive and negative impacts on introduced bacterial proliferation and persistence. Clay, nitrogen, organic

matter, and water-holding capacity where positively correlated, whilst $CaCO_3$ and sand texture were negatively correlated with bacterial survival. Soil type was identified as the main cause of increased N mineralisation when using a commercial BI (Laditi et al. 2012). The authors suggested the treated soil contained high levels of P_i, which reduced the N : P ratio, favouring bacterial (*Azospirillum brasilense*) growth and increasing N mineralisation. PMM did not significantly increase plant (*Zea mays*) P content within the same soil, although the experimental soil had 377 mg kg^{-1} P added, which may affect the plant P mobilising efficiency of the PMM (Laditi et al. 2012). Hart and Forsythe (2012) did find that single inoculation with *Rhizophagus irregularis* increased host (*Allium porrum* L. and *Plantago lanceolata* L.) nutrient content, irrespective of soil nutrient status, in contrast to mixed AM treatments. Similarly, using soil with high levels of P, Douds et al. (2007) were able to show a significant increase in total fresh weight of a potato crop using the single strain bio-resource Myke Pro SG2© (*Rhizophagus irregularis*); whilst Broschat (2009), using a low P soil, showed no significant effect of using four, multiple PGPM sp., BI products on Mexican fan palm (*Washingtonia robusta*).

Positive lab-based trial results of BI are often not replicated when applied at the field scale. As mentioned, there are many confounding factors that mask PGPM effects under suboptimal field conditions. For example, much of the experimental data using mycorrhizal inocula use single host plants and single strains of fungi. Under natural conditions, the initial carbon cost of fungal symbioses (which can be as high as 20% of plant photosynthate C (Smith, Read 2008)) to a seedling would be greatly reduced if the mycorrhizal fungi colonising the roots were part of a pre-existing common mycelial network connected to adjacent established plants (Hodge, Helgason & Fitter 2010). Also, sterile experimental conditions may impact AM colonisation strategies, due to the lack of ubiquitous soil bacteria which are known to affect AM colonisation rates (Dhillion 1992).

Soil disturbance, e.g. tillage, has been shown to exert a selection pressure on AM, with some genera, e.g. *Glomus*, being better adapted to soil disturbance than others such as *Gigaspora*, *Scutellospora* and *Racocetra* (Maherali, Klironomos 2007). *Gigaspora* and *Scutellospora* have been shown to form fewer intramycelial anastomoses (hyphal fusions) than *Glomus* (De La Providencia et al. 2005) and hence might be more susceptible to disturbance of the common mycelial network. Tillage also increases P_i and this increase impacts not just AM diversity, but also the abundance of AM structures; e.g. a study examining the effects of tillage and P-fertilisation found AM spore number positively correlated with both tillage and P-fertilisation, whilst hyphal density was negatively correlated (Sheng et al. 2013). BIs may also be

susceptible to loss of viability or reduced effectiveness if soil nutrient levels are too high, e.g. high concentrations of inorganic N fertiliser can be toxic to many microbes (Sarathchandra et al. 2001).

2.8. Conclusions

The inconsistency of BI performance and lack of independent validation does little to build confidence in their efficacy. This is compounded by the variations in experimental design, product design, carrier substrate, application rates and methods, and inoculum shelf-life. For commercial bio-resources to be effective, it is crucial to appreciate the complexity of the belowground interactions. Each soil environment encompasses an entire ecosystem and within each biome there exists a unique ecological web featuring multiple micro-environments (rhizosphere, mycor-rhizosphere). The complexity of the micro-environments is further exacerbated by the numerous interactions among bacteria and fungi, either competing for resources with the plant or facilitating presence and occurrence through modification of each micro-environment. Environmental variables (e.g. pH, moisture, temperature, redox conditions) place further constraints on rhizosphere processes that may affect microbial-soil-root interactions.

Compatible combinations of inoculated microbes (e.g. bacteria and AM) may enhance plant development within microsymbiont-legume systems. The synergistic / antagonistic effects of microbial inoculants within the plant-rhizosphere are a complex series of interactions, combined with native micro flora and abiotic environmental stress factors. A better understanding of the interactions between PGPM and their mode of action will allow for more efficient BI development.

Elucidating the complex tripartite relationship of plant, soil and PGPM would allow for more informed management practices. This is essential for the development of sustainable agriculture and soil conservation. For example, measuring soil and crop P requirements in combination with an accurate assessment of soil biota would lead to more accurate P recommendations, but also allow for a more appropriate bio-resource to be applied.

Increasing our knowledge of the dynamics of edaphic and biotic factors affecting soil biology will be central to the challenge of sustainable intensification of agriculture. Many questions need addressing, e.g. are there critical thresholds of microbial populations required to achieve

positive effects? Does increased bio-resource microbial diversity improve or hamper performance? How do bio-resources affect native soil biota, and vice versa? New areas of research are underway to examine these and other areas to unravel the complexities highlighted. Central to this is the inclusion of improved molecular techniques which will help refine data collection and synthesis, allowing for a more accurate phylogenetic analysis of PGPM. Success will be underpinned by biotechnological innovation, leading to strategic agricultural improvements, increasing yields, whilst being more ecologically sustainable.

2.9. References

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CHAPTER 3: ARTICLE I

Assessing the efficacy of commercial bio-inoculants on the yield of *Lolium perenne*: a laboratory study

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3.1. Abstract

Over the coming years, greater agricultural production will be needed to meet the demands of an increasing population, which will require increased phosphate fertiliser inputs, adding further pressures to finite rock phosphate reserves. The integration of biological inoculants (BIs) could reduce the need for inorganic fertiliser inputs through improving plant acquisition of soil macro- and micro-nutrients, such as phosphorus (P). Micro-organisms, both fungal and bacterial, contained within BIs improve plant nutrient acquisition through mechanisms such as plant root enhancement to the mobilisation of recalcitrant P fractions found within soil. However, there remains some ambiguity with regards to the efficacy of many commercial BI products. Some studies have found that positive plant growth responses could be traced to the non-living components of the applied BIs, as opposed to the microbial fraction, leading to scepticism of microbially based BI products, potentially undermining consumer confidence.

Two laboratory-based pot-trials were established to explore the efficacy of five commercial BIs. The first bioassay explored the effects of the BIs on *Lolium perenn*e growth and root development. Single inoculated grass plants were grown for ten weeks in a low P soil; root development and mycorrhizal fungal colonisation were then measured. The second bioassay examined the use of inert carrier substrates, found within many bio-inocula, and its effect on mycorrhizal colonisation, under varying nitrogen fertilisation regimes.

All tested BIs increased grass yields significantly, and while many BIs contain non-living additives, such as humates, treatments with living microbial fractions were found to have significantly more roots, leading to increased growth per unit P taken up by the grass. The second bioassay found the dual application of carrier media and mycorrhizal spores significantly increased grass yield, and the inert carrier media was a significant factor with respect to mycorrhizal root colonisation, increasing from 20% to 36%. There was also a positive correlation between nitrogen and fungal colonisation in the presence of the carrier media, which became a negative correlation when carrier media was absent. This is thought to be due to the enhanced cation exchange capacity of the carrier media capturing applied nitrogen which mycorrhizal fungi used to proliferate within the growth media and plant roots.

Overall, the study found positive results of bio-inoculant application on grass growth, but also highlighted the problematic nature of discerning the cause of increased plant growth and need to exercise caution when extrapolating laboratory-based results to field scale applications.

3.2. Introduction

A combination of an increasing population and reduced rock phosphate reserves poses a future problem for society. Greater agricultural production to meet the demands of an increasing population will require increased fertiliser inputs, or at least more efficient fertiliser usage. One method that could contribute to improved fertiliser use efficiency is the utilisation of micro-organisms. The integration of biological inoculants (BI) within integrated nutrient management systems aims to reduce inorganic fertiliser inputs through improving plant acquisition of soil macro- and micro-nutrients.

The global market for bio-inoculants is growing at an estimated rate of ~10% per annum (Berg 2009); valued at $440 million in 2012 and expected to reach $1,295 million by 2020 (Transparency Market Research 2014). Whilst there is a shortage of peer-reviewed studies reporting on the value of commercial BIs, the results that are available are varied, inconsistent and contradictory. There is no consensus on the efficacy of bio-inoculant products; of the few products which are demonstrated to have a clear (non-substrate induced) positive effect on plant (crop) growth, the mechanism of enhancement has not been fully elucidated (Owen et al. 2015).

3.2.1. Plant growth-promoting micro-organisms

The constituent plant growth-promoting micro-organisms used in BIs can be categorised into two main groups, bacteria and fungi. BIs often contain several genera of both to provide functional redundancy. Plant growth promotion is through a broad suite of growth enhancement mechanisms. One of the main proposed benefits of BIs is the potential to improve acquisition of essential plant nutrients such as nitrogen (N) and phosphorus (P).Within soil, the largely plant-unavailable recalcitrant pool of inorganic (P_i) and organic (P_o) phosphate can be exploited by BIs. Phosphorus efficiency ratio (PER) of applied BIs can be assessed by the measure of dry matter (DM) per unit P within the shoot biomass (g DM mg^{-1} P) (Hammond et al. 2009). A higher ratio is indicative of an improved conversion rate of P into DM and reduced P removal per unit yield from the soil; thereby helping to reduce the need for inputs of inorganic P fertiliser.

P-mobilising mechanisms mediated by bacteria include the production of enzymes, such as phosphatase (both alkali and acid) and phytase, siderophores (Franco-Correa et al. 2010) and

the lowering of pH through acid secretion (Illmer, Schinner 1992). For example, the P-mobilising potential of Actinobacteria has seen them exploited as BIs in which ligand exchange increases nutrient availability through the production of siderophores (Pragya, Yasmin & Anshula 2012).

The common free-living soil fungi, *Trichoderma* spp. have been shown to synthesise auxins which stimulate lateral plant root development (Benitez et al. 2004, Contreras-Cornejo et al. 2009). Several other fungi from the phylum Ascomycota are able to both solubilise P_i and mineralise P_o; e.g. *Aspergillus* and *Penicillium* spp. inhabit the plant root-rhizosphere and can secrete organic acids which mobilise P_i from rock phosphate, as well as phosphatase enzymes, allowing hydrolysis of P_o (Bolan 1991, Vassilev, Fenice & Federici 1996, Barrow, Osuna 2002).

Mycorrhizal fungi (MF) are another widely utilised fungal group used to improve plant yields; primarily through increased nutrient acquisition (Richardson et al. 2009, Richardson, Simpson 2011). The fungal mycelium effectively extends the plant root rhizosphere through forming an extensive network within the soil and leaf litter, which increases nutrient absorption potential. MF are particularly important for increasing the uptake of slowly diffusing ions such as PO_4^{3-}, and immobile nutrients such as Zn and Cu (Clark, Zeto & Zobel, 1999). The mycelial network is able to solubilise and mineralise P through the production of organic acids and enzymes, respectively (Marschner 1995, Koide, Kabir 2000). They have been shown to improve productivity in soils of low fertility, especially those low in P (Richardson, Simpson 2011, Smith et al. 2011). MF are obligate symbionts which can be grown only in the presence of host plants. Thus, BIs comprising them, generally contain preparations of spores propagated in pot cultures mixed with an inert carrier (Gentili, Jumpponen 2006).

3.2.2. Carrier substrate

Some commercial BIs contain as much as 99% inert substrate carrier as well as a consortia of MF. The carrier substrate of an inoculant provides a stable environment for microbial fractions, prolonging product shelf-life. They also act as a vector for dispersion or dissolution within the soil profile, post-application (Malusá, Sas-Paszt & Ciesielska 2012). Carriers can consist of soil materials (peat), clays (vermiculite and zeolite), organic materials (composts, coal) or inert materials e.g. perlite (Smith 1992, Malusá, Sas-Paszt & Ciesielska 2012). Liquid inoculants can be based on broth cultures, mineral or organic oils, or on oil-in-water suspensions (Malusá,

Sas-Paszt & Ciesielska 2012). A number of trials have actually found that the substrate carriers of some inoculants be more significant in promoting plant growth over the microbes they contain (Corkidi et al. 2004, Schenck zu Schweinsberg-Mickan, Müller 2009, Wiseman, Colvin & Wells 2009, Hale 2012).

Zeolite has been shown to improve the nutrient use efficiency of applied inorganic fertilisers (Ahmed et al. 2008, Aghaalikhani et al. 2012). Zeolites are naturally occurring alumino-silicate minerals with a cation exchange capacity of between 100 – 200 meq 100 g^{-1} (Barbarick, Pirela 1984). They possess channels in their structure thereby providing a large internal surface area for cation exchange; which can reduce ammonia volatilisation (Ahmed, Aminuddin & Husni 2006). The lattice structure of the inert zeolite provides protection for ammonium ions from nitrification (Ferguson, Pepper 1987), reducing potential N leaching (Ippolito, Tarkalson & Lehrsch 2011) by as much as 66% (Zwingmann et al. 2009). Attapulgite (magnesium aluminium phyllosilicate) is another common constituent of carrier substrates utilised in BI manufacturing. As with zeolite, it has a lattice structure, which not only protects NH_4 from volatilisation, but provides absorbent and water retention properties; which sees them marketed as soil conditioners.

3.2.3. Commercial bio-inoculants

In this study, a range of commercial BIs were assessed for their potential to increase growth of perennial ryegrass (*Lolium perenne* L.); with an emphasis on P mobilisation (bioassay 1). A second bioassay was established to examine the potential of an inert carrier media of a commercial BI to improve plant growth, by examining the effect of zeolite : attapulgite carrier on the colonisation rates of a mycorrhizal fungus (*Rhizophagus irregularis*) on *Lolium perenne* L. Five commercial BIs were tested (Fig. 3.1).

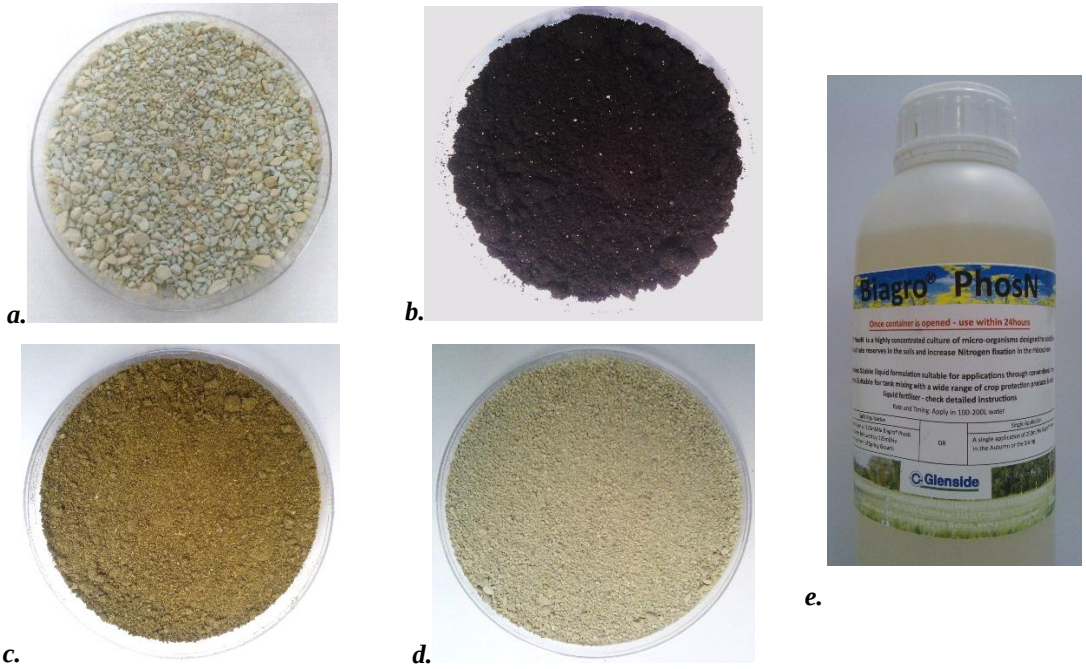

Figure 3.1. Commercial BIs *a)* Single Species Inoculant *b)* Biagro® S *c)* Biagro® MP *d)* Biagro® Grass *e)* Biagro® PhosN

A summary of each BI is provided in Table 3.1, though due to issues of intellectual proprietary it was not possible to ascertain the exact formulation of each product used.

Biagro® Grass (BG*)* is a vermiculite-based BI containing five MF (not specified) from the phyla, Glomermycota. The inoculum contains approximately 220,000 propagules kg^{-1} (although this can vary considerably). The infective component of the inoculum comprises of spores, roots and hyphae; lettuce is used as the host plant. Humates and bio-additives are also added (< 0.5% w:w). The product is targeted commercially at grass crops.

Biagro® S (BS) has a similar number of propagules as BG but are all spores, as opposed to root fragments as with BG. The formulation is not granular (Fig. 3.1b), which can limit seed dressing efficiency; as such it is regarded as a multi-purpose formulation for both grass and cereal crops. As with BG, there is a range of mycorrhizal species present, predominantly from the Glomus genus, together with support populations of beneficial free-living fungi and bacteria such as *Trichoderma* and *Bacillus* spp. respectively. Added nutrients and carriers consist of soluble seaweed extract, potassium humate, fulvates and amino acids.

Biagro® MP (BMP) is primarily aimed at cereals. Similar to BS, it contains both bacterial and fungal components; the formulation contains ~ 25 various micro-organisms, including *Bacillus* spp., rhizobia, azotobacter and a range of MF.

Biagro® PhosN (PN) is a liquid suspension and contains several phosphate mobilising and N fixing genera, *Bacillus, Paenibacillus, Azotobacter, Azospirillum* and *Beijerinckia.* The liquid medium comprises of water with mono-potassium phosphate and di-potassium phosphate.

Single Species Inoculant (SSI) is a bespoke inoculum formulated to contain one MF, *Glomus intraradices* (BEG 72). The carrier material is a zeolite : attapulgite mix (50 : 50). The BI contains root fragments and spores and the host plants were a combination of *Trifolium pratense* and *Zea mays.*

Table 3.1. Composition and recommended application rates of commercial bio-inoculants (BI) utilised in the trial

BI	**Composition**		**Rec. app. rate (kg ha^{-1})**
Biagro® Grass[z] (BG)	Granule	Consortium of five MF within a vermiculite carrier < 0.5% additives	1
Biagro® S[z] (BS)	Powder	Consortium of MF and bacteria with humates, algal extracts and amino acids	0.125
Biagro® MP[z] (BMP)	Powder	Consortium of MF and phosphate mobilising bacteria	0.150
Biagro® PhosN[z] (PN)	Liquid	Bacterial suspension containing phosphate mobilising bacteria and N fixing bacteria	250 ml ha^{-1}
Single Species Inoculant[y] (SSI)	Granule	*Glomus intraradices* (BEG 72) within an attapulgite : zeolite (50:50) carrier media	1

[z] Commercial products supplied by Glenside Group (Livingston, UK)

[y] Bespoke bio-inoculum manufactured by PlantWorks Ltd. (Sittingbourne, UK)

Zeolite has been shown to reduce nitrate leaching, with application rates of between 0.28 t ha^{-1} (MacKown, Tucker 1985) to 13.5 t ha^{-1} (Weber, Barbarick & Westfall 1984). It could be assumed that the low application rates of the BIs in this study will not have a significant effect on nitrate leaching (recommended application rates of both BG and SSI are 1 kg ha^{-1} which is ~ 950 g ha^{-1} of inert carrier media). However N has been shown to be positively correlated with mycorrhizal colonisation (Ali et al. 2009) and therefore it is possible that the microsites of the zeolite will provide N rich hotspots to allow MF sporulation and thus improve root colonisation of grass.

3.3. Aims

- To test the efficacy of five commercial bio-inocula on grass (*Lolium perenne* L.) growth (bioassay 1)
- To ascertain their mode of action with a focus on P uptake and root morphology
- To explore the effects a carrier media on grass growth and MF colonisation of grass (*Lolium perenne* L.) roots (bioassay 2)

3.4. Methodology

3.4.1. Growth media

A growth media with a low level of plant-available PO_4 and total P was used in both experiments (Table 3.2). The growth media consisted of sterilised sharp sand and sterilised topsoil (Homebase) at a ratio of 2 : 1 (~ 0.40 kg).

Table 3.2. Analysis[z] of the growing media – a 2 : 1 sand : soil steam sterilised mix. Electrical conductivity (EC) in µS cm^{-1},% organic matter (OM) measured as loss on ignition, macro- and micro-nutrients in mg kg^{-1} (Appendix 9.2.1-3)

			Plant -available (mg kg^{-1})			**Total (mg kg^{-1})**							
pH	**EC**	**OM**	**NO_3**	**NH_4**	**PO_4**	**P**	**K**	**S**	**Ca**	**Cu**	**Zn**	**Mn**	**Fe**
7.6	435	0.3	1.6	11.2	4	195	1996	460	8203	5	12	135	7072

[z] Growth media analysis determined as per standard protocols for EC, OM and pH. Extracts in 1M KCl analysed for NH_4^+ by the nitroprusside colorimetric method of Mulvaney (1996) and NO_3^- by the colorimetric Griess reaction of Miranda et al. (2001), PO_4-P extracted by sodium bicarbonate (Olsen, Cole & Watanabe 1954) and measured coliometrically (Murphy, Riley 1962). All other nutrients were measured by total reflection X-ray fluorescence (TXRF) (Appendix 9.1)

3.4.2. Bioassay 1

The first bioassay was established to explore the efficacy of five commercial BIs on grass growth and development. The experimental design consisted of single grass plants inoculated with commercial BIs and grown for ten weeks in growth units (Rootrainers™) with a 12.25 cm^2 surface area and 20 cm in depth (245 cm^3 volume) (Fig. 3.2), were filled up to 18 cm with the low P growing media (~ 350 g). *Lolium perenne* (Emorsgate seeds) seeds were surface sterilised (1 ml 70% ETOH / 0.1% Triton solution) and placed on moist blue roll and pre-germinated for five days. After five days, germinated seeds with similar radicle emergence were selected for the bioassay. A single grass seedling was planted in each growing unit and a BI treatment applied to the rooting zone, and then covered in more growing media (~ 50 g).

Figure 3.2. Rootrainers™ *a*) single book of four cells *b*) ten books placed in tray

The four commercial BIs and one bespoke BI were added at 400 times greater than the manufacturer's recommended application rates; this was due to the small volumes of the recommended application rates which would not have been practical for the surface area of the growth units (Table 3.3).

Table 3.3. Bio-inoculant (BI) recommended application rates and quantities used in trial. Quantities applied were ~ 400 times the recommended application rate per hectare (based on 20 kg ha^{-1} seed rate)

BI	Recommended application rate (kg ha^{-1})	Quantity applied (g)
BG	1	1
BS	0.125	0.125
BMP	0.150	0.150
PN	250 (ml ha^{-1})	250 µl
SSI	1	1

Treatments (non-sterilised) and a control were replicated five times (n = 5). Negative controls (sterilised) (n = 3) of each treatment consisted of autoclaved (121 °C) BI applied at the same rates as the non-sterilised treatments to account for any possible nutrient effects. The growth units (45 in total) were placed on a laboratory bench with artificial lighting (light intensity = 260 µmol m^{-2} s^{-1} PAR) with a minimum photoperiod of 16 h. Soil in the pots were maintained at 60% water holding capacity (WHC) (Appendix 9.2.4) by watering twice weekly. Soil water holding capacity was measured gravimetrically (Rowell 1994). To ensure P was the only limiting macro-nutrient, an adjusted Hoagland's nutrient solution with 80% reduced PO_4 was used (Appendix 9.3), with a total of 60 ml applied per growth unit over the course of the trial.

3.4.3. Bioassay 2

The second bioassay was set up to explore the effects of a commercial carrier media (zeolite : attapulgite (50 : 50)) on the potential to increase MF colonisation; and the effects of varying N application. The experimental design consisted of two factors, carrier media and N application. Sterilised spores of *Rhizophagus irregularis*[2], obtained from INVAM (West Virginia University), were viewed under a Zeiss microscope (Fig. 3.3) to examine for any contamination. Growth units, as per bioassay 1, were filled up to 19 cm with the growth media (~ 380 g). *Lolium perenne* (Emorsgate seeds) seeds were surface sterilised (1 ml 70% ETOH / 0.1% Triton solution), with each replicate receiving ~ 50 mg seed (20 kg ha^{-1}) (Cool et al. 2004). A 2 ml spore dilution (~ 50 spores) was applied to the seed, and the seed covered with more growth media (~ 20 g) (as recommended by supplier, INVAM). The zeolite : attapulgite (50 : 50) carrier treatment consisted of 1 g of sterilised substrate (sterilised by autoclaving at 121 °C for one hour) applied with the seed and / or spores.

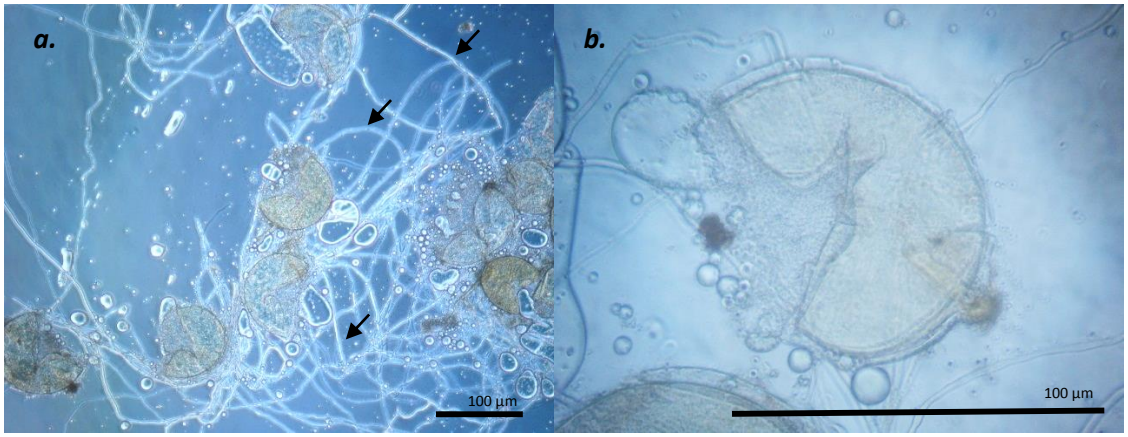

Figure 3.3. *a)* Spores of *Rhizophagus irregularis.* Arrows indicating subtending hyphae (×200 magnification) *b*) Individual spore (×400 magnification)

Treatments consisted of MF, carrier, MF + carrier and a control at three N fertilisation rates of zero N, low N and high N, three replicates giving a total of 36 growth units, maintained at 60% WHC. Plants were given reduced P Hoagland's solution (Appendix 9.3) adjusted for zero, low or high N every two weeks (equivalent to 11 kg ha^{-1} and 140 kg ha^{-1} total N applied, respectively). After two weeks, plants were thinned to ~ 10 plants per growth unit.

[2] Spores deposited by R. Francis at the University of Sheffield in 1995 (ID assigned G.3 LPA5)

3.4.4. CEC / AEC

The cation and anion exchange capacity of the zeolite : attapulgite carrier was measured using an un-buffered NH_4Cl solution. All exchange sites on the mineral were saturated with NH_4^+. KNO_3 was then applied to displace NH_4^+ from the exchange sites. NH_4^+ and Cl^- were then measured in the final extract (Grove, Fowler & Sumner 1982), corresponding to the CEC and AEC, respectively.

3.4.5. Bioassay measurements

3.4.5.1. Bioassay 1

After ten weeks, grass plants were harvested, shoot and root were separated and roots washed thoroughly in distilled water and floated out on water in transparent plastic trays, and scanned using a flatbed scanner (Perfection 4990 Photo; Epson Electronics America Inc., San Jose, CA, USA), a sub-sample taken of roots taken for fungal colonisation estimation (Section 3.4.6). The resulting image was processed using WinRhizo® software (Regent Instruments Inc., Canada) to determine the length of seminal and lateral roots per plant, and the resulting root system surface area. Six root classes were designated and the boundary conditions used by the software were from 0.1 mm to 0.5 > mm in diameter. Root classes > 0.2 mm were classified as seminal roots, and < 0.2 mm were classed as lateral roots (Zobel, 2009). After root length measurments both root and shoot dry matter (DM) measured (oven 80 °C / 24 hours). The DM was then dry-ashed (550 °C / 16 h) and the residue dissolved in 0.5 M HCl. The P content was then determined using the ascorbate / molybdate blue method (Murphy, Riley 1962).

3.4.5.2. Bioassay 2

After ten weeks, the grass was cut (Fig. 3.4) and oven dried to calculate DM and the P content of the biomass (as per Bioassay 1).

Figure 3.4. Growth units after ten weeks growth

3.4.6. Root staining

Roots of both bioassays were gently rinsed with distilled water to remove soil particles. Washed roots were then stored in a 50% ethanol solution. Root cells were cleared of cytoplasm using 2.5% KOH solution heated in a water bath (60 °C) for ~ 2 hours. After clearing, roots were rinsed three times with de-ionised water and then acidified using 2% HCl; this allowed the stain to adhere to any fungal structures present within the roots. Roots were stained using a Trypan blue stain (150 mg of Trypan blue, 100 ml de-ionised water, 100 ml glycerol and 100 ml lactic acid (80%)). Solutions were heated to 90 °C in a water bath for ~ 2 hours. The stain was decanted and excess stain within the roots removed by submerging in a de-staining solution (50% glycerol) at room temperature for 24 hours.

3.4.7. Fungal quantification

Roots were cut into ~ 1 cm lengths and 30 random root sections were placed on two glass microscope slides (7.5 cm × 2.5 cm), and fixed with a 50% glycerol solution and a cover slip placed over the top. Quantification was made using a Zeiss Axiophot microscope, at ×200 magnification. Fungal structures were scored on presence and intensity (Fig. 3.5) (Trouvelot, Kough & Gianinazzi-Pearson 1986). Assigned values were then uploaded to the Mycoclac program (www2.dijon.inra.fr) (Trouvelot, Kough & Gianinazzi-Pearson 1986). Total length colonised (Equation 1) and intensity of the colonisation (M%) were then analysed statistically.

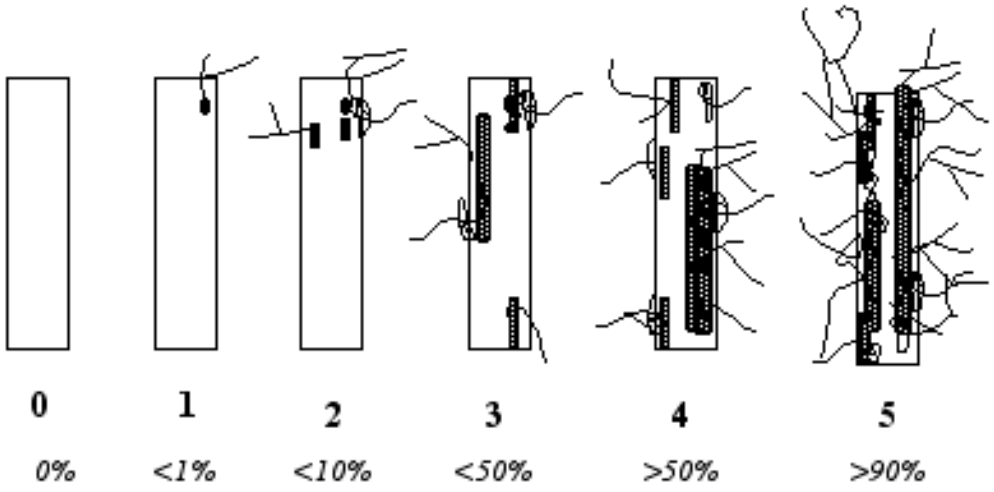

Figure. 3.5. Scoring roots for fungal colonisation. F% is a binary present or not present, M% quantifies the intensity of the fungi present (Trouvelot, Kough & Gianinazzi-Pearson 1986)

$$\mathrm{TRC} = \mathrm{F\%} * \mathrm{RL}$$

Equation1. Where TRC = total root colonised (cm), F% = frequency of colonisation and RL = total root length (cm)

3.4.8. Statistical analysis

Data was analysed for statistical significance with SPSS 22.0 (IBM). Percentage data was log transformed. All data was analysed for normality using Shapiro-Wilk test. Analysis of variance (Anova) with Fisher's LSD post hoc used to test for any significant treatment effects. Univariate analysis (SPSS) indicated any significant effects of multiple treatment factors of bioassay 2. Results were considered to be significant at the $p < 0.05$ level. All data was suitably transformed, if required, to conform with Levene's test of homogeneity.

3.5. Results

3.5.1. Bioassay 1

3.5.1.1. Elemental analysis of bio-inoculants

TXRF analysis (Appendix 9.1) of each BI revealed high levels of the macro-nutrients P, S and K for BMP and BS (Table 3.4).

Table 3.4. TXRF elemental analysis of bio-inoculants (BI), values are mg kg^{-1} (n = 3)

BI	P	S	Cl	K	Ca	Mn	Fe	Cu	Zn	Se
BG	411	40	408	14093	9627	253	7193	7	28	397
BS	1303	47631	11171	54230	4954	105	2522	28	59	448
BMP	3232	27473	10473	29351	5754	76	2033	9	32	409
PN	32	21	59	125	124	0.03	0.03	0.04	0.30	0
SSI	747	0	48	12502	9000	110	12195	8	42	370

3.5.1.2. Sterilisation process

There were no significant differences measured in the dry matter yields between the overall means of sterilised and non-sterilised BIs, 0.107 g and 0.125 g respectively (p = 0.186); an indication of the potential nutritive effect of the applied BIs (Appendix 9.4.4). Only one BI, PN, yielded significantly less shoot dry matter after being sterilised, 0.129 g compared with 0.018 g post sterilisation (Fig. 3.6), PN was also found to contain the least amount of all measured elements within the TXRF analysis (Table 3.4). There was a significant difference between sterilised and non-sterilsed BIs on root dry matter (p = 0.02), 0.055 g and 0.071 g respectively. There was however a significant interactive effect between BI and sterilisation treatment ($p < 0.001$) (Appendix 9.4.3); in which sterilisation effects on root DM varied between BIs (Fig. 3.6). For example, both PN- and BMP-treated grass had significantly more root DM than their respective sterilised treatments, a possible indication that the sterilisation process prevented microbially stimulated root growth (Appendix 9.4.2 for full root class breakdown); whereas BS-treated grass had significantly more root DM post sterilisation.

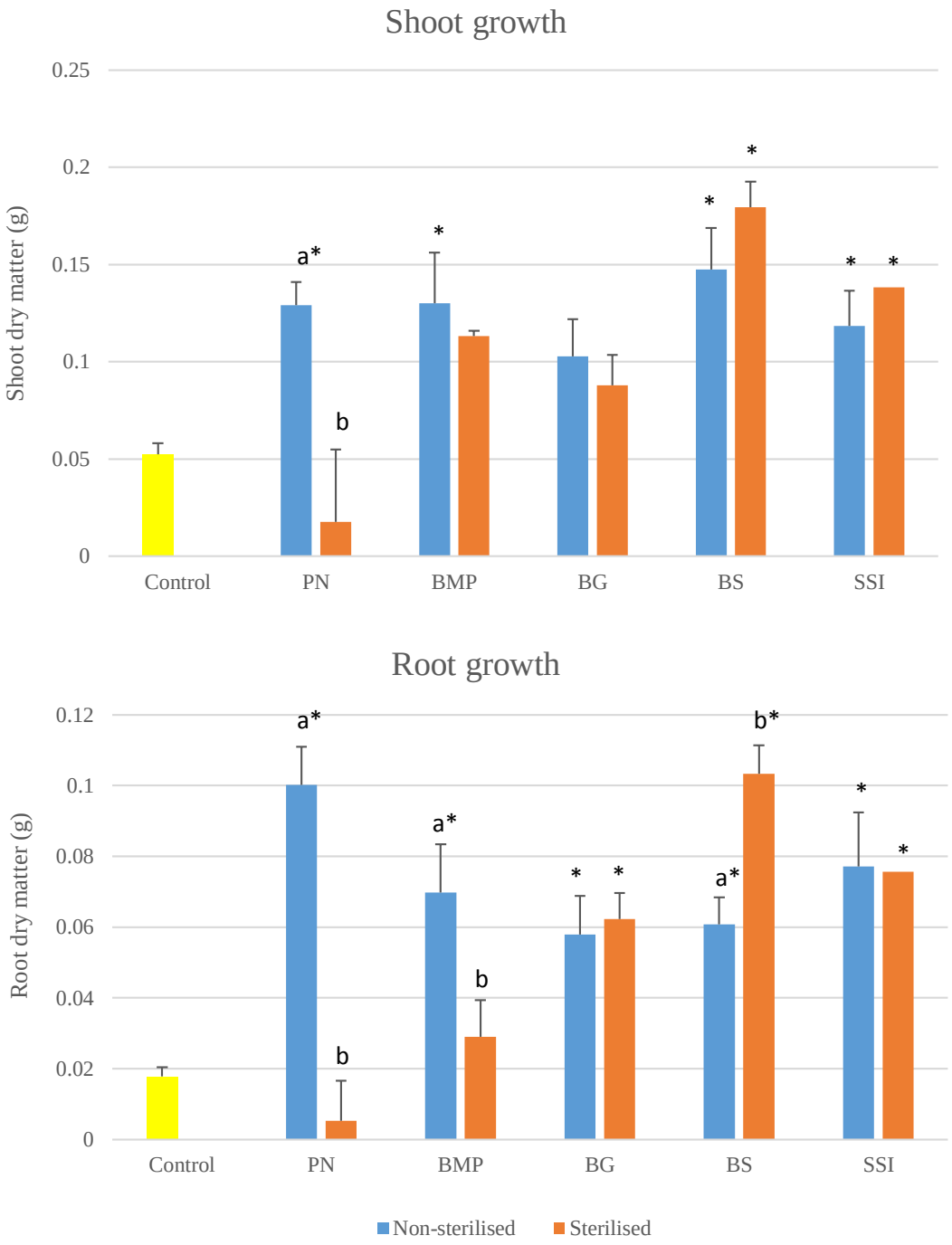

Fig 3.6. Shoot and root dry matter (g) of sterilised (n = 3) and non-sterilised (n = 5) BI-treated grass. Error bars are ± SEM. Different letters represent a significant difference of sterilisation within each BI (T-test, $p < 0.05$). Asterisk indicates any significant difference between treated (BI) and non-treated (Control) plants (T-test, $p < 0.05$). PN was significantly affected by sterilisation with both shoot and root growths reduced, BMP yielded significantly reduced root growth after sterilisation. BS exhibited increased root growth after sterilisation

3.5.1.3. Plant growth and P uptake

The following results focus on the non-sterilised treatments and control, hereafter referred to as treatments. The control yielded the least shoot and root DM; with PN, BMP, BS and SSI-treated grass all yielding significantly higher shoot DM (Fig. 3.6). The BG-treated grass also yielded more than twice the control, although this was not statistically significant. Similarly, root DM was significantly higher for all BI-treated grass (Fig. 3.6).

There was a significant treatment effect on % P content of the shoot DM ($p = 0.034$), with PN- and BS-treated grass significantly lower than the control; although shoot P (mg), was significantly higher only for BMP- and SSI-treated grass only (Table 3.5), suggesting a BI-mediated P-effect from both BMP and SSI. The % P within the root DM was significantly lower for all the BI treatments ($p < 0.0001$), but with significantly greater root mass (Fig. 3.6) resulted in both PN- and SSI-treated grass having significantly more root P (mg). The PER were all higher than the control, significantly so for PN- and BS-treated grass, which would indicate a potential benefit of the BIs in improving the conversion of P taken up into shoot DM (Table 3.5).

Table 3.5. Shoot and root % P content, total phosphorus (P) content (mg) and phosphorus efficiency ratio (PER) (g DM mg^{-1} P) of bio-inoculant (BI) treated grass. Values in parenthesis are ± 1 standard deviation. Values with an asterisk indicate significant difference as compared to the control ($n = 5$) (Anova, LSD post hoc, $p < 0.05$)

	Shoot			**Root**	
BI	**% P**	**Total P (mg)**	**PER (g DM mg^{-1} P)**	**% P**	**Total P (mg)**
Control	0.20 (± 0.03)	0.104 (± 0.004)	0.50 (± 0.08)	0.096 (± 0.004)	0.017 (± 0.004)
PN	0.11* (± 0.01)	0.143 (± 0.03)	0.92* (± 0.11)	0.056* (± 0.010)	0.055* (± 0.007)
BMP	0.17 (± 0.03)	0.220* (± 0.08)	0.59 (± 0.09)	0.037* (± 0.006)	0.026 (± 0.011)
BG	0.15 (± 0.04)	0.148 (± 0.04)	0.67 (± 0.14)	0.041* (± 0.007)	0.024 (± 0.011)
BS	0.12* (± 0.02)	0.182 (± 0.06)	0.82* (± 0.17)	0.033* (± 0.008)	0.019 (± 0.003)
SSI	0.18 (± 0.07)	0.206* (± 0.07)	0.60 (± 0.17)	0.049* (± 0.023)	0.037* (± 0.021)

3.5.1.4. Root zone P exploitation

BI treatments significantly increased total root length 3-4 fold (Table 3.6); all root classes measured were significantly higher than control (Appendix 9.4.1). The ratio of lateral roots (measured as the total of root class < 0.1 and < 0.2) to seminal roots (measured as the total of root class < 0.3, < 0.4, < 0.5 and > 0.5) was significantly higher for BG-treated grass. Increased root density coupled with significantly higher surface areas saw the amount of soil exploited by each grass plant significantly increased by the BI treatments,; with PN notably high for both variables. The result of increased rooting zones reduced the amount of P taken up per unit surface area; a significant reduction for all BI-treated grass (Table 3.6).

Table 3.6. Root characteristics of bio-inoculant (BI) treated roots. Total root length (cm), Specific root length (SRL), ratio of lateral to seminal growth (L: S), density of roots per unit soil volume (RLD), surface area (SA) and phosphorus (P) uptake per unit surface area (P. SA^{-1}). Values in parenthesis are ± 1 standard deviation. Values with an asterisk indicate significant difference as compared to the control (n = 5) (Anova, LSD post hoc, $p < 0.05$)

BI	Total root length (cm)	SRL	L : S	RLD (cm L cm^{-3} soil)	SA (cm^{-2})	P. SA^{-1} (µg.cm^{-2})
Control	641 (± 16)	3.8 x10^4 (± 1.0 x$10^{4)}$	2.85 (± 0.13)	2.62 (± 0.07)	33.66 (± 1.91)	3.59 (± 0.08)
PN	2504* (± 219)	2.5 x10^4 (± 2.8 x10^3)	2.22 (± 0.26)	10.22* (± 0.89)	163.99* (± 20.92)	1.21* (± 0.16)
BMP	1886* (± 721)	2.8 x10^4 (± 5.1 x10^3)	2.69 (± 0.39)	7.70* (± 2.94)	108.69* (± 44.90)	2.54* (± 1.15)
BG	1937* (± 573)	3.6 x10^4 (± 1.1 x10^4)	3.49* (± 0.58)	7.90* (± 2.34)	97.72* (± 33.47)	1.81* (± 0.28)
BS	1695* (± 275)	2.9 x 10^4 (± 4.7 x10^3)	3.07 (± 0.42)	6.92* (± 1.12)	89.42* (± 11.86)	2.27* (± 0.73)
SSI	2230* (± 825)	3.1 x10^4 (± 6.7 x10^3)	2.77 (± 0.46)	9.10* (± 3.37)	122.79* (± 48.18)	2.01* (± 0.37)

3.5.1.5. Mycorrhizal colonisation

All the BI treatments successfully colonised roots. Staining revealed several fungal structures associated with mycorrhizal colonisation (Fig. 3.7).

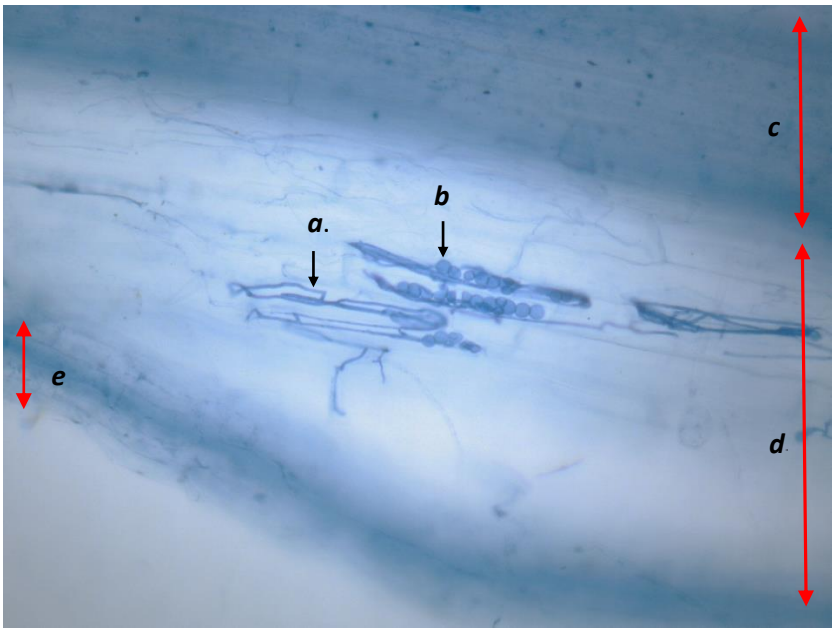

Figure 3.7. Stained root fragment showing fungal structures (picture from BMP treatment) (×200 magnification), (*a*) intercellular fungal hypha (*b*) vesicles (*c*) stele (*d*) root cortex (*e*) epidermis

The total root length colonised (TRC) of the BS treatment was significantly lower than all other treatments (Anova, $p < 0.05$) whilst the intensity of the colonisation (M%) of the SSI treatment was significantly higher (Fig. 3.8).

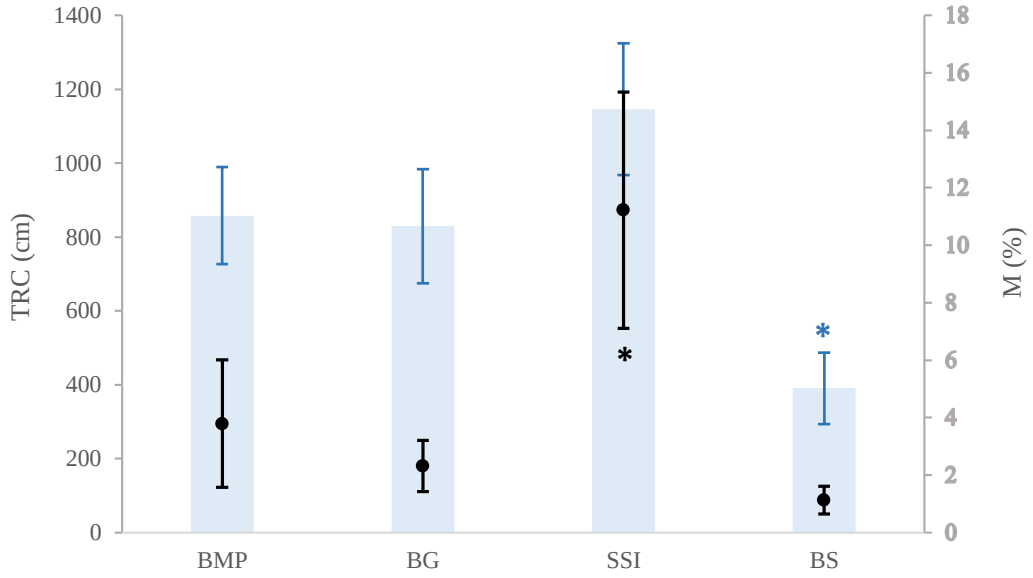

Figure 3.8. Total colonised root (TRC) length (cm) (Bar) and the Intensity (M%) of the colonisation (●). Control roots had no colonisation (data not shown). PN was not included as it contains no MF. Statistically significant results shown by asterisk, TRC (*), M% (**), (n = 5) (Anova, LSD post hoc, $p < 0.05$). Error bars are ± 1 SEM

3.5.2. Bioassay 2

Both the application of N and *Rhizophagus irregularis* spores had a significant effect on shoot and root DM (Table 3.7). Furthermore, there was an interactive effect between the two treatments (Table 3.8). High N application reduced root DM when fungi were not present (Table 3.7). The carrier significantly reduced shoot DM compared with the control, and was shown to be a significant factor with respect to fungal colonisation ($p = 0.012$, Table 3.8), mean colonisation measured at 36% with carrier, compared to 20% without. Furthermore there was an interactive effect between N application and carrier, in which increased N increased colonisation in the presence of the carrier media (Table 3.8.

Table 3.7. Shoot and root dry matter (DM) (g), frequency of mycorrhizal colonisation (F%) and intensity of colonisation (M%) at three rates of N application (zero, low, high) of *Lolium perenne* grass roots treated with combinations of *Rhizophagus irregularis* (fungi) and carrier media ($n = 3$). Values in parenthesis are ± 1 standard deviation. Different superscript letters indicates significant difference within each N treatment ($n = 3$) (Anova, LSD post hoc, $p < 0.05$). Asterisk indicates significant increase for F % or M % within each N treatment (Anova, $p < 0.05$)

	Shoot DM (g)			**Root DM (g)**			**F%**			**M%**		
Treatment	**Zero**	**Low**	**High**	**Zero**	**Low**	**High**	**Zero**	**Low**	**High**	**Zero**	**Low**	**High**
Control	0.043^{a} (± 0.137)	0.112^{a} (± 0.015)	0.196^{ac} (± 0.088)	0.052^{a} (± 0.015)	0.107^{a} (± 0.028)	0.078^{ab} (± 0.015)	-	-	-	-	-	-
Carrier	0.027^{b} (± 0.007)	0.068^{b} (± 0.025)	0.074^{b} (± 0.024)	0.032^{a} (± 0.007)	0.112^{a} (± 0.078)	0.047^{a} (± 0.007)	-	-	-	-	-	-
Fungi	0.023^{b} (± 0.002)	0.132^{ac} (± 0.016)	0.140^{c} (± 0.020)	0.039^{a} (± 0.016)	0.231^{b} (± 0.009)	0.191^{bc} (± 0.143)	46.3* (± 6.4)	3.7 (± 3.2)	11.1 (± 5.6)	18.5* (± 6.5)	3.5 (± 3.0)	1.7 (± 2.2)
Fungi +Carrier	0.058^{a} (± 0.003)	0.150^{c} (± 0.014)	0.247^{a} (± 0.036)	0.133^{b} (± 0.038)	0.219^{b} (± 0.021)	0.330^{c} (± 0.075)	27.8 (± 9.6)	35.2* (± 23.1)	45.4* (± 7.1)	1.6 (± 0.5)	3.5 (± 4.2)	2.5 (± 0.2)

Table 3.8. *p* values of a univariate statistical analysis. Individual factors and interactive effects were considered significant at the $p < 0.05$ level

Factor	Shoot DM	Root DM	F%	M%
Carrier	0.528	0.240	0.012	0.073
Nitrogen	< 0.001	< 0.001	0.057	0.033
Fungi	< 0.001	< 0.001	n / a	n / a
*Nitrogen*Fungi*	0.076	0.048	n / a	n / a
*Carrier*Fungi*	< 0.001	< 0.001	n / a	n / a
*Carrier*Nitrogen*	0.048	0.306	0.002	0.009
*Carrier*Nitrogen*Fungi*	0.078	0.029	n / a	n / a

A significant interactive effect was observed between carrier and N, and carrier with fungi on shoot DM (Table 3.8), whilst there were also significant interactive effects between N and fungi, carrier and fungi and all three treatment factors on root DM (Table 3.8).

3.6. Discussion

BI additions to soil can increase plant yields through several mechanisms, from the stimulation of root growth, thereby increasing nutrient and water uptake; to increasing the uptake of key plant nutrients through acquisition and delivery mechanisms e.g. fungal hyphae (Marschner 1995, Koide, Kabir 2000). The majority of studies conducted with BIs have focussed on higher-value crops rather than grass. However, grasslands are the overwhelming land-use type in large proportions of the country, and are likely to play an increasingly important role in food production. This study aimed to explore the potential yield gains, if any, of a range of commercially available BIs applied to grass.

3.6.1. Bioassay 1

All BI treatments increased shoot DM compared to the control, (PN, BMP, BS and SSI yielding significantly higher) (Fig 3.6). Shoot P was also significantly higher for two BIs (Table 3.5); however, the percentage P content of the shoot DM was slightly reduced. Root DM showed a significant reduction in the percentage P content (Table 3.5). These results suggest that the BIs increased the mobilisation of P from the growth medium, and that this extra P was effectively converted to shoot DM.

The root system is entirely responsible for acquisition of water and nutrients from soil. The limited mobility of P within soil creates diffusion gradients and the spatial configuration of a root system is a key component in acquiring nutrients (Walk, Jaramillo & Lynch 2006). An efficient root system architecture is essential for a plant to maximise growth at minimum cost. Changes in root system architecture are a response to resource allocation economics in which the lower construction cost of smaller root classes, coupled with increased nutritive adsorption potential, makes them a sound investment by plants when nutrients are poorly available (Ragothama 1999, Vance, Uhde-Stone & Allan 2003).

There was a significant increase in all root classes of the BI-treated plants over the control (Appendix 9.4.1). Adventitious rooting contributes to efficient P acquisition, however the metabolic investment retards the development of other root classes, as seen in the control plants, and may leave the plant susceptible to other stresses e.g. water stress through reduced basal root length (Walk, Jaramillo & Lynch 2006). In soils with limited nutrient availability,

the root architecture can adapt so as to increase total adsorptive surface area (López-Bucio, Cruz-Ramírez & Herrera-Estrella 2003). The use of BIs can stimulate lower order root development thus increasing root adsorptive surface area, increasing the potential P adsorptive area per unit root growth (Drew, Saker 1978). Within this study, the BIs significantly increased all root class orders, resulting in significantly higher surface areas. BIs were shown to increase the potential P adsorption area, and reduce the amount of P adsorbed per unit root surface area, thereby potentially reducing the P depletion zone surrounding roots. Direct P uptake by the roots may have been reduced further as fungal-derived P constitutes the majority of P making up plant P status (Smith et al. 2011). The smaller roots of the control plants, relying on direct P uptake only, were likely to exhaust the limited P supply within a short distance of the root. Limited P availability has been shown to reduce plant root systems depth and network convex area (Ingram et al. 2012). This, coupled with the slow replenishment of the rooting zone with soluble P (Marschner 1995), may have contributed to the reduced yield.

The findings of this study mirrored others in which root growth was significantly increased by BIs when grown in soils of low P availability (Baffoni et al. 2012). There was a significant reduction in the % P of root DM with the addition of BIs. This could be a reduction in the partitioning of P from leaves to sink organs when nutrient supply stress is removed. When nutrient supply is low, phloem transport of P_i from older leaves to sink organs is used by plants to maintain PER; the gene Pht1;5 plays a critical role in mediating the mechanism (Lewis et al. 2011). Low P_i induces sugar dependent systemic expression of genes which modulate the root system architecture (Lewis et al. 2011). The results of this study found the control grass roots to contain a significantly higher % of P compared to the BI treatments. Furthermore, the nutrient stress alleviated by the BI treatments, resulted in a significantly higher PER for both PN- and BS-treated grass.

Similarly, the SRL of the treatments was found to be lower than that of the control, although not significantly so. MF fungi have been shown to significantly decrease SRL (Berta et al. 1995) due to several factors, chiefly, increased root diameter and density, and an increase in inter- and intra-cellular constituents, such as fungal structures from mycorrhizal colonisation (Berta et al. 1995).

3.6.1.1. Colonisation

All fungal inoculants featured in the trial successfully colonised *Lolium perenne* roots, with % colonisation rates ranging from 22% (BS) to 57% (SSI). This trial used *Lolium perenne*, a C3 grass, which has a reduced mycorrhizal dependency than C4 plants (Wilson, Hartnett 1997). Fungal colonisation of C3 plants is further reduced by the fibrous nature of the root system (Hetrick, Kitt & Wilson 1988, Hetrick, Wilson & Leslie 1991) but also the photosynthetic pathway utilised is less P-demanding than within C4 plants (Bueckert 2013). In spite of this, the C3 *Lolium perenne* had similar colonisation rates as that of the C4 *Zea mays* (Corkidi et al. 2004), a standard plant used for mycorrhizal inoculum assays (INVAM, 2008). The growth media in both trials differed in nutrient availability, PO_4 for example, which was 12 mg kg^{-1} (Corkidi et al. 2004) compared to 4 mg kg^{-1} for this trial. Variation in colonisation frequencies could be due to the low P growth media, both promoting and restricting colonisation, depending on the P sensitivity of the BI. Wiseman et al. (2009) observed reduced fungal colonisation of *Zea mays* and suggested that the high P content of the growth media used (89 mg kg^{-1}) inhibited fungal colonisation.

3.6.1.2. Nutritive content

Many studies examining the efficacy of applied BIs did not use sterilised treatments, and therefore any nutritive effects cannot be ascertained (Baffoni et al. 2012, Laditi et al. 2012). This particular study did indicate a potential nutritive effect from the carrier substrate of the BIs. For example, with the exception of PN, shoot DM was un-affected by sterilisation. Macro- and micro-elemental analysis of the BIs revealed them to contain large concentrations of P, S and K and interactive effects were observed for PER, in which sterilised BMP and SSI exhibited higher PER than their non-sterilised counterparts, whilst sterilised PN was lower. Furthermore, the sterilisation process may have seen a flush of nutrients released from cell lyses. Several other studies have shown a similar trend, in which treatment effects could be traced back to the non-living components of the applied BIs (Schenck zu Schweinsberg-Mickan, Müller 2009, Wiseman, Colvin & Wells 2009).

However there were significant impacts on several measured variables on non-sterilised treatments (Appendix 9.4.4), notably below-ground parameters, suggesting yield gains were driven by different biologically-mediated mechanisms. Non-sterilised treatments were shown to have significantly more root DM than their sterilised counterparts. PN and BMP were

notably higher than their respective sterilised treatments, a possible indication of microbially stimulated root growth.

An interactive effect between sterilisation and BI was observed, for example PN and BMP, both of which contain bacterial fractions, had several root class orders reduced by sterilisation. The heat sterilisation would have been enough to prevent the microbial fraction from stimulating plant growth; bacteria are known to induce changes in root system architecture, for example, the stimulation of lateral root growth, chiefly through the synthesis of plant growth hormone, auxin (Oláh et al. 2005).

BS showed the opposite trend, with both shoot and root DM decreasing in non-sterilised treatments, the latter being significant. The nutritive additions within BS, algal extract and potassium humate for example, may have increased soil fertility and as such the non-sterilised BS treatment may have reduced yields due to a plant carbon cost exerted by the microbial fraction. Cell lyses of the biological fraction during the sterilisation process may also have added to soil fertility, as discussed previously.

Although similar yield gains were achieved from the application of sterilised BIs as there were for non-sterilised due to potential nutritive inputs, there were advantages seen when non-sterilised BIs were applied, such as a significant increase in root DM. Such influences may bring plant benefits not tested within this trial, for example water stress alleviation and pathogen resistance.

This study was conducted on an extreme of low plant-available P (~ 4 mg kg^{-1}). Within the field, this would have been supplemented with additional inorganic fertiliser, and increased P_o availability. This study focused only on one yield cut; subsequent yield cuts may have revealed further differences as the P is further exhausted. For example, the significant differences in PER between sterilised and non-sterilised BI may change as the nutrient flush provided by sterilised treatments is exhausted. The changes in root morphology of the un-sterilised BI-treated grass may then begin to provide an advantage such as enhance nutrient acquisition and thus increased PER. If true, it may be postulated that the application of BIs may improve grass utilisation of applied inorganic fertiliser.

3.6.2. Bioassay 2

Zeolite has been shown to retain NH_4 due to the narrow channels within the mineral lattice protecting it from nitrification (MacKown, Tucker 1985). It is widely used by farmers, especially in Australia, to increase fertility of soils as the high CEC of the mineral helps prevent leaching of the applied N fertilisers. Furthermore, increased retention of NH_4 by zeolite could potentially lead to increased mycorrhizal colonisation, in one study colonisation rates were found to be ~ 10% higher with NH_4-N (Leigh, Fitter & Hodge 2011), in which MF were shown to preferentially transport NH_4 over NO_3 to host plant when N was not limiting (Leigh, Fitter & Hodge 2011).

The CEC of the zeolite used within this study measured 51.6 (± 1.1) (meq 100 g^{-1}), however, may have actually contributed to reducing yields in the absence of fungi. The zeolite treatment was applied in the top two cm of the growth unit and may have restricted the flow of applied N to the rooting zone. The zeolite also measured 0.5 (± 0.04) (meq 100 g^{-1}) for AEC, which may have similarly restricted the flow of essential macro- / micro-nutrients to the rooting zone.

Mycorrhizal hyphal growth and sporulation have been shown to be positively correlated with mineral fertiliser application when soils contained increased organic matter (OM) (Gryndler et al. 2001). This was explained by the OM increasing retention of applied inorganic fertiliser, whilst also being a source of organic N and P. Within this trial, the OM of the soil was very low and the zeolite increased nutrient retention and, in effect, became a proxy for OM, contributing to increased fungal colonisation and subsequent grass yield gains.

The amount of N applied had an effect on the percentage root colonisation, with highest and most intense colonisation occurring within the zero N and no zeolite treatment. There are several reports of both positive (Saleh et al. 1998, Jha, Netra & Saxena 2005) and negative (Termorshuizen, Ket 1990, Bethlenfalvay, Andrade & Azcon-Aguilar 1997) effects of N fertiliser on mycorrhizal colonisation. Increased colonisation under nutrient stress could be due to changes in root morphology (Tingey, Johnson & Phillips 2005, Schalamuk et al. 2006), for example, increased lateral branching (Zhang, Forde 1998), which stimulates fungal root colonisation through increased root exudates (Tisserant, Gianinazzi & Gianinazzi-Pearson 1996). Whilst colonisation increased this did not result in concurrent increases in yield, a potential indication of the carbon cost of maintaining the fungal symbiosis in low nutrient status soil.

3.7. Conclusion

This study was able to show effective mycorrhizal colonisation by commercial BIs applied to a *Lolium perenne* grass. BIs were shown to significantly increase the rooting of the grass, increasing the nutrient acquisition ability of the grass. The study also highlighted the difficulty of separating microbial-derived positive plant growth promotion from the effects of other non-living components of various BI formulations. The findings of laboratory-based trials cannot be fully extrapolated to field-scale applications due to the potential that the controlled conditions may bias results. For instance, nutritive effects seen in the laboratory would not necessarily have as significant effect in the field, due to the reduced recommended application rates.

This study did highlight the positive effects of zeolite on fungal colonisation, suggesting the inclusion of this inert mineral, or similar materials, in BI design which feature mycorrhizal fungi would enhance the colonisation potential of the BI. Future experiments will explore the impacts of the application of BIs within a field situation so that results are more easily extrapolated to real-world scenarios.

3.8. References

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CHAPTER 4: ARTICLE II

Investigating P mobilisation of various phosphate complexes using commercial bio-inoculants

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4.1. Abstract

Rock phosphate is a fundamental component of inorganic fertiliser manufacture, which is integral to agricultural production. Global demand for fertiliser, hence rock phosphate, has increased, placing pressure on this essential finite resource. Plant beneficial micro-organisms, found naturally within soil, have been isolated and used to create a range of plant growth-promoting biological inoculants to maintain or increase agricultural production whilst reducing the need for inorganic fertiliser application. Strategies mediated by micro-organisms for increasing the availability of plant nutrients, such as phosphorus, include solubilisation and mineralisation of recalcitrant phosphorus pools found in soil. The solubilisation and mineralisation potential of five commercial bio-inoculants were investigated, using two insoluble sources of P, tri-calcium phosphate and phytate. All five bio-inoculants tested were found to increase the availability of phosphate from the two recalcitrant P-sources. P was liberated in greater quantities with tri-calcium phosphate, which was accompanied with a marked drop in solution pH. A laboratory-based pot trial investigated the phosphorus efficiency ratio, a measure of the dry matter per unit P within the shoot biomass of *Lolium perenne* following application of two inorganic fertilisers of varying solubility, triple super phosphate and the less soluble rock phosphate. Two of the tested bio-inoculants were found to exhibit P mediated growth gains in the form of increased yield, total shoot P, and phosphorous efficiency ratio; though this was dependent on the P source. Yield and shoot P gains were found to be mediated by differing fractions of the living component of each BI dependent on P source. This study indicates that commercial bio-inoculants have the potential to increase crop yields, but that further work is needed to understand their mode of action and the factors that determine their effectiveness.

4.2. Introduction

The importance of phosphorus (P) for crop production is well documented, indeed, there are estimates that between 40 – 60% of current crop yields are due to inorganic fertiliser applications (Stewart et al. 2005, Roberts 2009). However, much of the P applied to agricultural land in the past is now stored in the soil as surplus P. This soil 'legacy P' store supplements the native soil reserves, slowly released by weathering, and represents a potentially valuable and under-utilized P resource that could be used to reduce applications of costly inorganic (manufactured) fertilisers without affecting crop yields (Sattari et al. 2012, Sharpley et al. 2013, Withers et al. 2014).

The availability of soil P for plant-uptake is influenced by the physical, chemical and biological properties of the soil and the root characteristics of plants. For example, effective exploration of soil by plant roots, root exudates and associations with microorganisms are factors which contribute towards soil-P mobilisation mediated through biochemical processes at the root / soil interface (rhizosphere). Plant-P requirements are dependent on an adequate supply of P into solution for plant uptake, especially during early growth (Grant et al. 2001). As P availability in soils is inherently low, plant demand must be met either through increased inorganic fertiliser addition or improving soil P acquisition strategies (Ramaekers et al. 2010). One strategy for improving the availability of native and legacy P in the soil is the use of biological inoculants (BI).

4.2.1. Phosphorus application and mobilisation

The use of inorganic P fertilizers, such as di-ammonium phosphate (DAP) and triple superphosphate (TSP), to supplement soil P is commonplace in commercial agriculture. The effectiveness of these fertilisers varies according to soil type, soil pH and soil P status; for example fertiliser P mobility is low in calcareous and alkaline soils (Mclaughlin, Alston & Martin 1988). Application of P fertiliser, and some manures, causes an initial sharp rise in solution P, at the point of soil contact (Allen, Mallarino 2006). Chemical equilibrium between P in soil and P in solution is rapidly established through soil P adsorption and precipitation of lower solubility P compounds. With time, soil solution P moves into less available, non-labile, pools (Yang et al. 2012). The portion of applied P fertiliser that remains in labile (plant-available form) as measured by standard soil P tests is relatively small, around 13% (Johnston et al. 2014). The portion of applied that remains in less labile and more recalcitrant forms

depends on soil chemical and mineralogical properties, P uptake by crops, P movement through the soil profile, and soil erosion (Allen, Mallarino 2006). The more recalcitrant P complexes of native and legacy P are associated with highly-weathered acidic soils rich aluminium and iron oxyhydroxides, and in calcareous soils where P is present as, or transformed into, insoluble calcium compounds such as apatites (Stevenson, Cole 1999). Rock phosphate (RP), the base raw material of TSP and DAP production, offers a cheaper source of P for crops and can be applied directly to soil, although due to its low solubility is generally better suited to more acidic soils. The availability of P from RP is often too low to demonstrate an immediate impact on crop production; however, studies have shown increased crop yields when RP application was combined with PMM (Blal et al. 1990, Vessey 2003, Salimpour et al. 2010, Prasad et al. 2012).

The rhizosphere of plants contains a plethora of micro-organisms which increase plant growth through many mechanisms, including increasing plant-available P in the soil. Many organisms have been isolated, from the rhizosphere and soil, and exploited commercially. Micro-organisms mobilise P by several mechanisms including solubilisation, mineralisation and translocation (Owen et al. 2015). In particular, organisms contained within BI are able to mobilise the more recalcitrant P fractions of native and legacy soil P, through exudation of P-mobilising compounds such as organic acids, protons and enzymes (e.g. phosphatases and phytases); mechanisms of P release include, (*i*) complexing and chelating cations bound to P, (*ii*) competing with P ions for sorption sites, (*iii*) dephosphorylation of organic P and (*iv*) by acidifying the rhizosphere, directly dissolving mineral-P (by proton extrusion) (Chen et al. 2006, Richardson et al. 2009, Oburger, Jones & Wenzel 2011). The P released which is not used for their own growth becomes available for plant-uptake.

The main bacterial genera utilised within commercial BI formulations include *Bacillus* and *Pseudomonas* with *Aspergillus, Penicillium, Trichoderma*, *Mucor* and *Mortierella* making up the root-associated fungi (RAF) (Khan et al. 2010). Mycorrhizal fungi (MF) are another group of P-mobilising organisms exploited commercially. MF are able to form an extensive network of hyphae (extraradical mycelium) in the soil which acts as an extension of the plant's roots system, increasing surface area to volume ratio for nutrient uptake; which also secretes enzymes and organic acids to mobilise P (Marschner, Dell 1994, Koide, Kabir 2000). Whilst they are obligate symbionts, the spores of MF can contain endo-symbiotic bacteria which are also able to mobilise P (Cruz et al. 2008).

Five commercial BIs, Biagro® Grass (BG), Biagro® MP (BMP), Biagro® S (BS), Single Species Inoculant (SSI) and a bacterial inoculant Biagro® PhosN (PN) were examined for their ability to mobilise P from two recalcitrant P complexes, tri calcium phosphate (TCP) and phytate. A bioassay was also established to assess the phosphorus efficiency ratio (PER) of the BIs, supplemented with two phosphate fertilisers of varying P-availability, TSP and RP. Phosphorus efficiency ratio (PER) of applied BIs, is a measure of the potential of BIs to improve nutrient acquisition through reducing plant metabolic costs, for example, reduced partitioning of P from shoots to roots; PER of applied BIs can be assessed by the measure of yield per unit P within the shoot biomass (g DM mg^{-1} P) (Hammond et al. 2009). A higher ratio is indicative of an improved conversion rate of P taken up into dry matter; reduced P removal per unit yield from the soil, thereby helping to reduce inorganic P fertiliser applications.

4.3. Aims

- To measure the solubilisation and mineralisation potential of five commercial bio-inoculants

- To assess the potential of these bio-inoculants to improve the phosphorus efficiency ratio of applied inorganic P fertilisers

4.4. Methodology

4.4.1. P-liberation trial

The phosphate mobilising potential of five BIs was determined by estimation of PO_4 in solution after incubation in a nutrient broth (Section 4.4.1.2) containing a recalcitrant phosphate complex. For a measure of the solubilisation potential tri-calcium phosphate ($Ca_3(PO_4)_2$, TCP) was used, and phytate ($C_6H_{18}O_{24}P_6$) was used as a measure of mineralisation potential. Each BI was placed in a 50 ml centrifuge tube with 20 ml of broth which was adjusted to pH 7 (Vijayaraghavan, Primiya & Gnana 2013).

4.4.1.1. Bio-inoculants (BI)

The BI used within both trials (TCP and phytate) are listed in Table 4.1. The quantity of BI applied was the same for both trials, and were relative to each application rate, to enable comparisons between BIs. BIs were added at 400 times greater than the manufacturer's recommended application rates; this is not a true reflection of the recommended application rates in a field setting (Table 4.1), however, such rates would be unworkable for the surface areas and volumes used within both trials.

Table 4.1. Bio-inoculant (BI) recommended application rates and quantities used in trial. Quantities applied were ~ 400 times the recommended application rate per hectare (based on 20 kg ha^{-1} seed rate)

BI	Recommended application rate (kg ha^{-1})	Quantity applied (g)
Biagro® Grass[z] (BG)	1	1
Biagro® S[z] (BS)	0.125	0.125
Biagro® MP[z] (BMP)	0.150	0.150
Single Species Inoculum[y] (SSI)	1	1
Biagro® PhosN[z] (PN)	250 (ml ha^{-1})	250 µl

[z] Commercial products supplied by Glenside Group (Livingston, UK)
[y] Bespoke bio-inoculum manufactured by PlantWorks Ltd. (Sittingbourne, UK)

4.4.1.2. Pikovskaya's broth (PB)

PB, modified for P solubilisation detection (Pikovskaya 1948, Rao 1995), was used as both, a substrate for the solubilisation estimation, and as agar for plates to visualise P solubilising colonies. The PB contained nutrients for microbial growth and a recalcitrant phosphate (Table 4.2). Yeast extract in the medium provided nitrogen and other nutrients necessary to support bacterial and fungal growth and glucose acted as an energy source. The broth was sterilised by autoclaving at 120 °C for one hour.

Table 4.2. Composition of broth and agar media

	g l^{-1}
Yeast extract	0.5
Glucose	10
Calcium phosphate / phytate	2.5 / 1
Ammonium sulphate	0.5
Potassium Chloride	0.2
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Iron (II) sulphate	0.0001
Agar	15

4.4.1.3. Pikovskaya's agar

Micro-organisms grow on the agar and form a clear zone around a colony, due to phosphate solubilisation in the vicinity of the colony. The nutritional composition of the agar was identical to the broth except with the addition of agar (Table 4.2). The media was heated to boiling and autoclaved (121 °C) for 15 minutes to sterilise, before 20 ml was poured into sterile petri dishes. The same amount of inoculum as per the solubilisation / mineralisation trial was applied to the surface of the agar (Table 4.1).

4.4.1.4. Incubation

Caps were loosely placed on the tubes to allow air passage. Tubes were placed on an orbital shaker (150 rpm) and incubated at 30 ± 1 °C for 7 days (n = 3). After the seven day incubation period the cultures were centrifuged (7000 g, 10 min) and 5 ml supernatant aliquots were filtered through Whatman no.42 filter paper to remove thick polysaccharide-like exudates and the filtrate diluted (100 : 1); PO_4 in solution was then determined using the ascorbate /

molybdate blue method (Murphy, Riley 1962). Microbial P solubilisation was also visualised on agar plates. BIs were also incubated in de-ionised (DI) water to establish any background P present within the BI (n = 3). The pH of all the samples was measured after incubation.

4.4.1.5. P estimation

Values obtained from BI water treatments were used to establish any P present within BI i.e. P associated with BI. Controls consisted of broth media and P source (no BI) i.e. P associated with P source. P-liberation by BIs was quantified using:

$$P_{tot} = P_{BI} - P_{water} - C$$

P_{tot} = P_i total

P_{BI} = P_i in broth + BI,

P_{water} = P_i in water + BI,

C = P_i in broth + P source (TCP or phyate)

4.4.2. P-source bioassay

The bioassay was established to assess the efficiency of BI uptake of two P fertilisers of varying solubility. Some micro-organisms present within BIs, such as MF, require time to establish within plant roots (Corkidi et al. 2004). As such, P fertilisers were applied six weeks after seeding (after the first harvest). Growth chambers with a 12.25 cm^2 surface area and 20 cm in depth (245 cm^3) (Rootrainers ™), were filled up to 18 cm (~350 g) with growth media (Table 4.3). *Lolium perenne* (Emorsgate seeds) seeds were surface sterilised (1 ml 70% ETOH / 0.1% Triton solution). Seed (~ 50 mg, equivalent to 20 kg ha^{-1} (Cool et al. 2004)) was dressed with a BI treatment (Table 4.1) and covered with more growth media (~50 g).

Table 4.3. Analysis[z] of the growth media. 2:1 sand : top soil, both steam sterilised. Electrical conductivity (EC) in µS cm^{-1}, % organic matter (OM) measured as loss on ignition, macro- (plant-available) and micro-nutrients (total) in mg kg^{-1}

			Plant -available (mg kg^{-1})			**Total (mg kg^{-1})**							
pH	EC	OM	NO_3-N	NH_4-N	PO_4-P	P	K	S	Ca	Cu	Zn	Mn	Fe
7.6	435	0.3	1.6	11.2	4	195	1996	460	8203	5	12	135	7072

[z] Growth media analysis determined as per standard protocols for EC, OM and pH. NH_4^+ was determined by the nitroprusside colorimetric method (Mulvaney 1996) and NO_3^- by the colorimetric Griess reaction (Miranda, Espey & Wink 2001), PO_4-P by sodium bicarbonate extraction (Olsen, Cole & Watanabe 1954). All other nutrients were measured by total reflection X-ray fluorescence (TXRF) (Appendix 9.1)

The five BI treatments and a control were replicated 12 times (n = 12), a total of 72 growth units. The growth trays were placed on a laboratory bench with artificial lighting (light intensity = 260 µmol m^{-2} s^{-1} PAR) with a minimum photoperiod of 16 h. Soil in the pots was maintained at 60% of its water holding capacity by watering twice weekly. Soil water holding capacity was measured gravimetrically (Rowell 1994). Two weeks after seeding, units were thinned to ~ 10 plants. To ensure P was the only limiting macro-nutrient, an adjusted Hoagland's nutrient solution (Appendix 9.3), with 80% reduced PO_4, was applied every two weeks to ensure good crop establishment. Six weeks after seeding grass was cut to ~ 1 cm above soil surface (first cut) (Appendix 9.5.1), the shoot dry matter (DM) and % P content were measured (section 4.4.2.1). After the first cut, P treatments were applied (TSP, RP and 0P) (n = 4) at rates equivalent to 100 kg P_2O_5 ha^{-1} (44 kg ha^{-1} P), as per recommended application rates for a soil of such P-status in RB209 (Defra. 2010). Phosphate treatments consisted of TSP and RP with a P_2O_5 content of 46% and 15% respectively (Appendix 9.5.2). Hoagland's nutrient applications thereafter contained no phosphate. After a further six weeks whole plants were harvested.

4.4.2.1. Bioassay measurements

Shoots and roots were separated and shoot dry weights measured (oven 80 °C / 24 hours). The dry matter was then dry-ashed (550 °C / 16 h) and the residue dissolved in 0.5 M HCl, the P content of the shoot biomass was then determined using the ascorbate / molybdate blue method (Murphy, Riley 1962).

4.4.2.2. Colonisation estimation

After gently rinsing with DI water to remove soil particles, roots were stored in a 50% ethanol solution. Root cells were cleared of cytoplasm using 2.5% KOH solution heated in a water bath (60 °C) for ~ 2 hours. After clearing, roots were rinsed three times with DI water and then acidified using 2% HCl, allowing the stain to adhere to any fungal structures present. Roots were stained using Trypan blue (150 mg of Trypan blue, 100 ml DI water, 100 ml glycerol and 100 ml lactic acid (80%)). Solutions were heated to 90 °C in a water bath for ~ 2 hours. Stain was decanted and excess stain within the roots removed by submerging in a de-staining solution (50% glycerol) at room temperature for 24 hours. Roots were cut into ~ 1 cm lengths and 30 random root sections were placed on two glass microscope slides (7.5 cm × 2.5 cm), and fixed with a 50% glycerol solution and a cover slip placed over the top. Quantification was made using a Zeiss Axiophot microscope, at ×200 magnification. Fungal structures were scored on presence and intensity (Trouvelot, Kough & Gianinazzi-Pearson 1986). Assigned values were then inputted into the Mycocalc (www2.dijon.inra.fr) program (Trouvelot, Kough & Gianinazzi-Pearson 1986).

4.4.3. Statistical analysis

Data were analysed for statistical significance with SPSS 22.0 (IBM). Percentage data was log transformed. All data was analysed for normality using Shapiro-Wilk test. Univariate analysis indicated interactive effects between factors, P-source and BI. Analysis of variance (Anova) identified any significant results within each factor, post hoc, Fisher's LSD, tested for any significant individual treatment effects. Kruskal-Wallis (KW) one-way analysis of variance employed if data was not normally distributed. Results were considered to be significant at the $p < 0.05$ level. All data was suitably transformed, if required, to conform with Levene's test of homogeneity. Interactive effects between factors were calculated by comparing group means after controlling for a covariate (reported as estimated marginal means).

4.5. Results

4.5.1. P-liberation

After seven days of incubation, there was a marked drop in pH for all BI treatments within the TCP broth (Table 4.4). All BI treatments significantly reduced the solution pH of the TCP broth compared to the controls (Broth + P source (TCP or phytate)) ($p < 0.001$), which remained close to starting pH of 7. There was no significant change in pH for the phytate broth. Considerably less P was mobilised from the phytate than the TCP. There was significantly ($p < 0.001$) more PO_4 liberated from TCP by the BG, BS, BMP, SSI and PN treatments (Table 4.4). BS, SSI and PN liberating significantly ($p < 0.001$) more PO_4, 54,76 and 58 mg l^{-1} respectively, than the control, when phytate used as P-source (Table 4.4).

Table 4.4. PO_4 (mg l^{-1}) liberated into solution from two phosphate sources (TCP 0.25% w/w and phytate 0.1%). Controls consist of the broth plus P source (TCP or phytate) and no BI. PO_4 values of each BI are the total minus PO_4 measured in DI water of each respective BI. Values in parenthesis are ± 1 standard deviation. Different superscript letters represent significant differences ($n = 3$) (Anova, LSD post hoc, $p < 0.05$)

	TCP		**Phytate**	
BI	**pH**	**PO_4 (mg l^{-1})**	**pH**	**PO_4 (mg l^{-1})**
Control	6.7^{a} (± 0.1)	68^{e} (± 13)	6.7 (± 0.1)	37^{a} (± 14)
BG	4.3^{b} (± 0.4)	437^{ab} (± 141)	6.5 (± 0.1)	45^{a} (± 2)
BS	4.3^{b} (± 0.03)	164^{d} (± 19)	6.6 (± 0.1)	54^{b} (± 5)
BMP	4.2^{b} (± 0.04)	328^{bc} (± 63)	6.6 (± 0.2)	19^{c} (± 4)
SSI	3.4^{c} (± 0.6)	309^{c} (± 27)	6.4 (± 0.1)	76^{c} (± 15)
PN	4.2^{b} (± 0.2)	490^{a} (± 68)	6.5 (± 0.2)	58^{b} (± 7)

Cultured agar plates revealed several different species of both fungi and bacteria present within the various BI (Fig.4.1). As well as the stated mycorrhizal fungi (as per the label), which were not visualised on plates, non-mycorrhizal fungi were observed in BG and BS (Fig. 4.1*a*,*b* and *c*); BS also showed signs of bacterial colonies forming. SSI appeared to be dominated by one fungus, which a morphological examination suggests to be *Mortierella* sp. (Fig. 4.1*d*). Both BMP and PN saw bacterial colonies forming, the latter harbouring several different bacterial colonies (Fig. 4.1*e*, *f*).

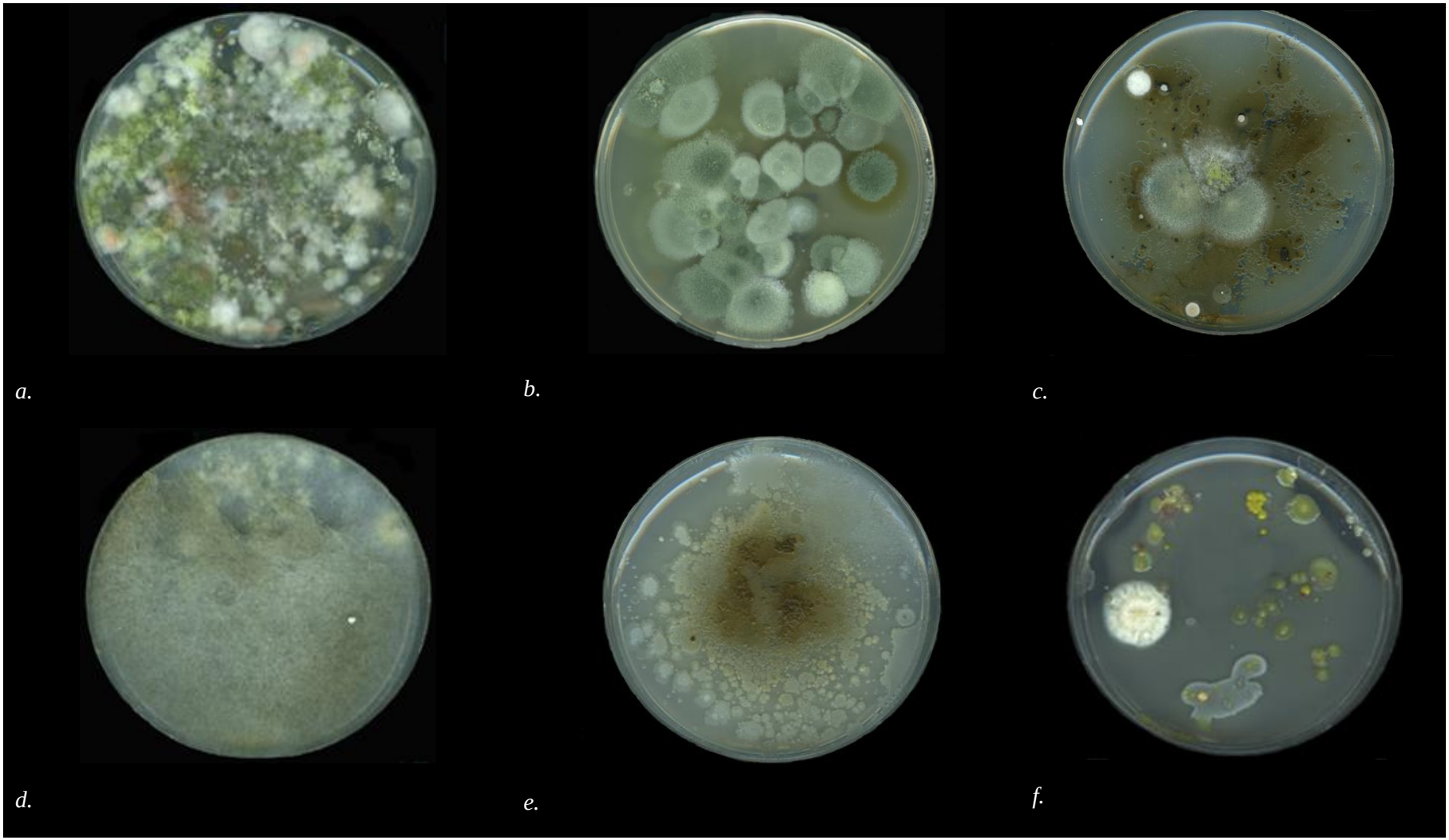

Figure 4.1. Agar plates after seven days incubation. Following inoculation with *a*) Biagro® Grass, *b*) Biagro® Grass, *c*) Biagro® S, *d*) Single Species Inoculum, *e*) Biagro® MP, *f*) Biagro® PhosN

4.5.2. Phosphate-source

Six weeks post-seeding there were no significant differences in DM yield. BMP-treated grass had significantly greater % P content ($p = 0.014$) and total shoot P (mg) ($p = 0.015$), and whilst yields were not statistically significant, they were over two fold greater than the control, suggesting a potential P effect of BMP-treated grass (Table 4.5). The PER was found to be significantly higher ($p = 0.04$) for BS (0.88 g DM mg^{-1} P) than the control (0.70 g DM mg^{-1} P) (Table 4.5), but with no significant difference in % P or total shoot P.

Table 4.5. Dry matter (DM) yield (g), percentage P content of shoot (% P), total shoot P (mg) and phosphorus efficiency ratio (PER) (g DM mg^{-1} P) of grass ($n = 12$) after six weeks growth prior to P fertiliser treatments. Values in parenthesis are ± 1 standard deviation. Different letters represent significant differences (Anova, LSD post hoc, $p < 0.05$)

BI	DM (g)	% P	Total shoot P (mg)	PER (g DM mg^{-1} P)
Control	0.027 (± 0.005)	0.13^{b} (± 0.08)	0.045^{b} (± 0.015)	0.70^{b} (± 0.11)
BG	0.027 (± 0.026)	0.17^{ab} (± 0.02)	0.047^{b} (± 0.036)	0.72^{b} (± 0.10)
BS	0.042 (± 0.028)	0.14^{b} (± 0.01)	0.059^{b} (± 0.036)	0.88^{a} (± 0.06)
BMP	0.071 (± 0.037)	0.22^{a} (± 0.02)	0.154^{a} (± 0.061)	0.54^{b} (± 0.06)
SSI	0.054 (± 0.019)	0.18^{ab} (± 0.03)	0.101^{ab} (± 0.040)	0.67^{b} (± 0.12)
PN	0.041 (± 0.018)	0.20^{ab} (± 0.03)	0.079^{ab} (± 0.022)	0.61^{b} (± 0.08)

P treatments were applied after the first cut, and left for a further six weeks, after which a destructive harvest was taken. Both treatment factors (BI and P treatment) were found to have a statistically significant effect on all measured variables. Interactive effects between factors were also observed (Table 4.6). For example, total shoot P and yield were significantly greater for BMP-treated grass when RP was applied, and BS-treated grass when TSP applied (Table 4.7).

Table 4.6. Univariate analysis of both factors (P treatment and bio-inoculant) and potential interactive effects. Results considered significant at $p < 0.05$ (marked with asterisk)

	DM	% P	Total shoot P	PER
Bio-inoculant	< 0.0001*	< 0.0001*	0.006*	< 0.0001*
P treatment	0.025*	< 0.0001*	< 0.0001*	< 0.0001*
*Bio-inoculant * P treatment*	< 0.0001*	0.002*	< 0.0001*	0.002*

Treatment of grass with BG almost doubled total shoot P (mg) between first and second cuts (pre-P treatment and the no P treatment), from 0.047 mg to 0.085 mg, but although greater than the control was not significant. However, the second cut (post-P treatments) BG-treated grass yield (0.155 g) was significantly greater than the control (0.085 g) within the no P treatment, the PER was also significantly increased (1.81 g DM mg^{-1} P) the control (1.33 g DM mg^{-1} P). Conversely, SSI was found to increase total shoot P accumulation (0.092 mg) but did not significantly increase yield, resulting in a non-significant PER (Table 4.7).

The significant % P and total shoot P (0.22% and 0.154 mg) of BMP-treated grass in the first cut (pre P-treatment) (Table 4.6), was replicated with RP application. BMP-treated grass yield (0.155 g) and total shoot P (0.133 mg) were both significantly greater than the control (0.089 g, 0.068 mg respectively) when RP was applied. The PER however was found to be significantly reduced (1.16 g DM mg^{-1} P) compared to the control (1.32 g DM mg^{-1} P) (Table 4.7).

BS (0.218 g) and SSI (0.155 g) treated grass yielded significantly greater than the control (0.072 g) with TSP application. The BI-treated grass biomass exhibiting significantly reduced % P content (0.21 and 0.17 respectively) than the control (0.36%). However, BS significantly increased both total shoot P and PER (0.423 mg and 0.52 g DM mg^{-1} P) compared to the control (0.257 mg and 0.28 g DM mg^{-1} P) (Table 4.7), whilst SSI saw no significant increase in total shoot P (0.255 mg) but did have a significantly higher PER (0.60 g DM mg^{-1} P).

Table 4.7. Phosphate treatments (Zero P, TSP, RP) applied with five bio-inoculant (BI) treatments and controls. Dry matter (DM) yield (g), P content of biomass (% P), total shoot P (mg) and P efficiency ratio (PER) (g DM mg^{-1} P). Values in parenthesis are ± 1 standard deviation. Different letters represent significant difference between bio-inoculants within each P treatment (n = 4) (Anova, LSD post hoc, $p < 0.05$)

P treatment	BI	DM (g)	% P	Total shoot P (mg)	PER (g DM mg^{-1} P)
Zero P	Control	0.09^{b} (± 0.03)	0.076^{a} (± 0.004)	0.06^{bc} (± 0.02)	1.33^{b} (± 0.07)
	BG	0.16^{a} (± 0.05)	0.06^{b} (± 0.01)	0.09^{ab} (± 0.02)	1.81^{a} (± 0.28)
	BS	0.09^{b} (± 0.02)	0.076^{ab} (± 0.005)	0.06^{bc} (± 0.01)	1.33^{b} (± 0.08)
	BMP	0.13^{ab} (± 0.03)	0.07^{ab} (± 0.01)	0.09^{ab} (± 0.02)	1.41^{b} (± 0.15)
	SSI	0.12^{ab} (± 0.01)	0.08^{a} (± 0.01)	0.09^{a} (± 0.01)	1.30^{b} (± 0.10)
	PN	0.09^{b} (± 0.02)	0.07^{ab} (± 0.01)	0.06^{c} (± 0.02)	1.54^{ab} (± 0.27)
RP	Control	0.09^{bcd} (± 0.02)	0.08 (± 0.01)	0.07^{bc} (± 0.02)	1.32 (± 0.11)
	BG	0.09^{bc} (± 0.01)	0.067 (± 0.002)	0.06^{bc} (± 0.01)	1.48 (± 0.05)
	BS	0.07^{d} (± 0.01)	0.078 (± 0.003)	0.05^{c} (± 0.01)	1.28 (± 0.05)
	BMP	0.16^{a} (± 0.03)	0.09 (± 0.01)	0.13^{a} (± 0.02)	1.16 (± 0.11)
	SSI	0.11^{b} (± 0.01)	0.07 (± 0.01)	0.08^{b} (± 0.01)	1.42 (± 0.17)
	PN	0.08^{cd} (± 0.01)	0.09 (± 0.03)	0.07^{bc} (± 0.02)	1.27 (± 0.50)
TSP	Control	0.07^{c} (± 0.01)	0.36^{a} (± 0.02)	0.26^{a} (± 0.04)	0.28^{a} (± 0.02)
	BG	0.09^{c} (± 0.02)	0.28^{bc} (± 0.03)	0.27^{a} (± 0.07)	0.36^{a} (± 0.05)
	BS	0.22^{a} (± 0.06)	0.21^{de} (± 0.06)	0.42^{b} (± 0.05)	0.52^{b} (± 0.09)
	BMP	0.099^{c} (± 0.002)	0.26^{cd} (± 0.03)	0.26^{a} (± 0.03)	0.38^{a} (± 0.04)
	SSI	0.16^{b} (± 0.04)	0.17^{e} (± 0.02)	0.26^{a} (± 0.02)	0.60^{b} (± 0.09)
	PN	0.09^{c} (± 0.01)	0.33^{ab} (± 0.01)	0.30^{a} (± 0.08)	0.30^{a} (± 0.01)

There was a significant BI effect on both the overall mean F% and M% ($p < 0.001$ and 0.01, respectively) on grass roots; P-source was found to have no significant effect on the overall mean of either colonisation parameters. However, there were interactive effects between BI and P-source (Figures 4.2, 4.3). BS-treated grass had the highest colonisation frequency when no P was applied (65.6%), colonisation dropping with increasing P availability (43.3% (RP), 27.8% (TSP)), conversely BG and SSI exhibited the opposite trend (Table 4.8).

Table 4.8. Mycorrhizal colonisation frequency (F%) and the intensity of the colonisation (M%) of grass roots inoculated with BI. Values in parenthesis are ± 1 SEM. Different letters represent significant difference between bio-inoculants (n = 3) (Anova, $p < 0.05$)

P treatment	BI	F%	M%
Zero P	*BG*	0^{e}	0^{bc}
	BS	65.6^{a} (± 25.1)	42.8^{a} (± 20.1)
	BMP	21.1^{cd} (± 8.7)	1.5^{c} (± 1.3)
	SSI	22.2^{bcd} (± 6.2)	4.2^{bc} (± 2.1)
RP	*BG*	1.1^{e} (± 1.1)	0.8^{bc} (± 0.8)
	BS	43.3^{abc} (± 15.0)	12.0^{ab} (± 7.6)
	BMP	11.1^{de} (± 6.8)	0.8^{c} (± 0.8)
	SSI	30.0^{abcd} (± 11.7)	2.4^{bc} (± 1.0)
TSP	*BG*	28.9^{abcd} (± 2.2)	8.3^{ab} (± 2.8)
	BS	27.8^{abcd} (± 11.3)	0.7^{c} (± 0.5)
	BMP	2.2^{e} (± 2.3)	0.02^{c} (± 0.02)
	SSI	56.7^{ab} (± 16.5)	3.7^{bc} (± 1.7)

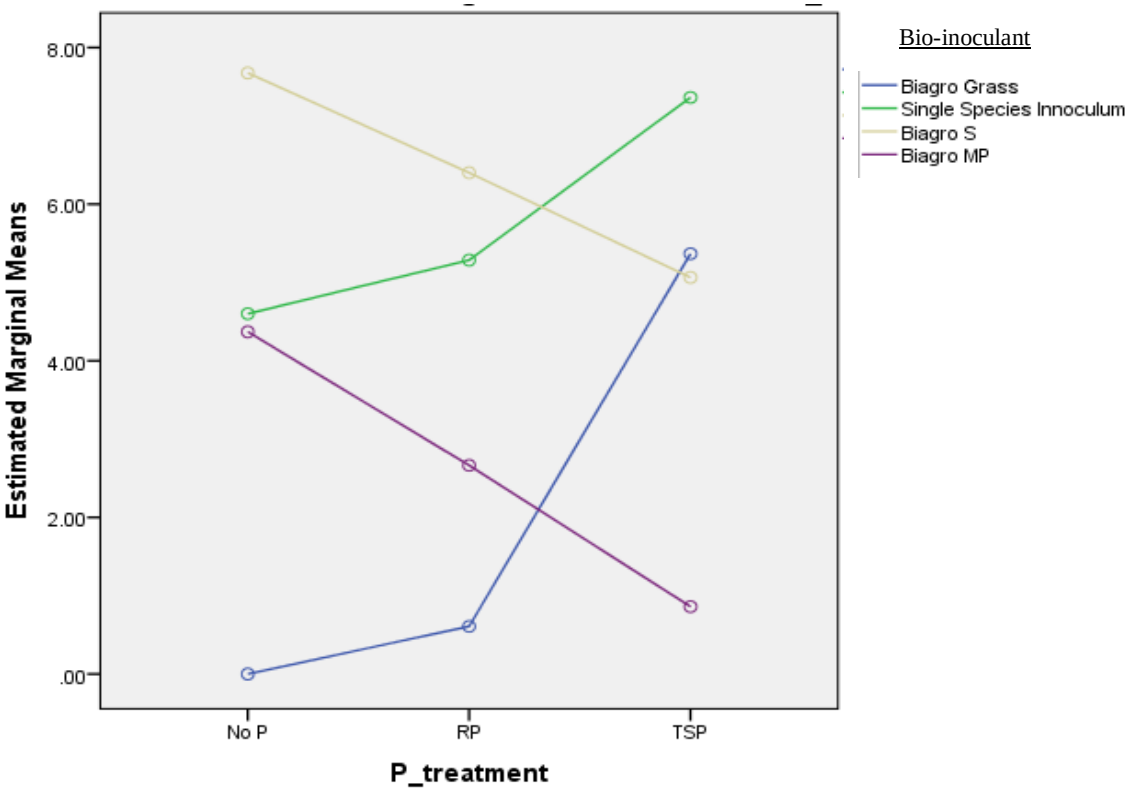

Figure 4.2. Interaction between P-source and BI on mycorrhizal colonisation (F%) (Univariate, $p = 0.003$)

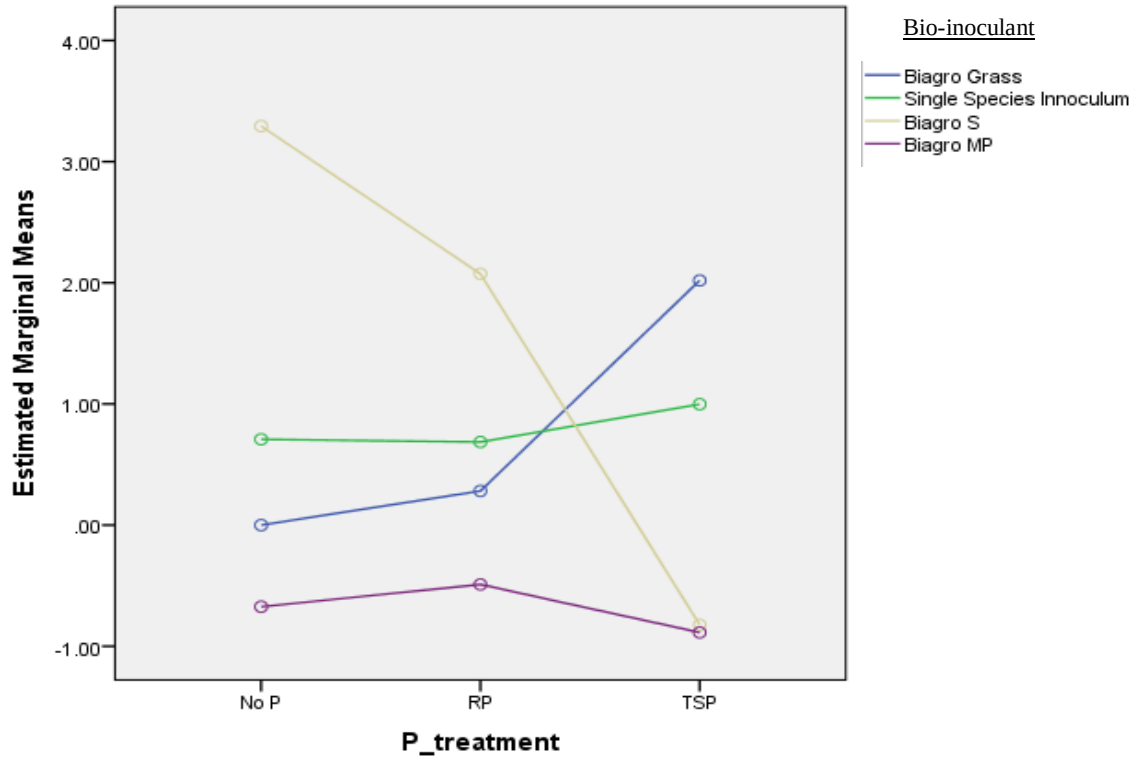

Figure 4.3. Interaction between P-source and BI on the intensity of mycorrhizal colonisation (M%) (Univariate, $p = 0.014$)

Microscopic examination of fungi present within the grass roots, revealed several morphological differences between fungi of each BI treatment, and within each P treatment. F% of BG-treated grass with no P applied was measured at 0%, however roots were extensively covered in black dots, RAF (Fig. 4.4). The appearance of the RAF was seen on both, BG and SSI, but appeared to be reduced with the application of RP and TSP (Fig. 4.5, 4.6).

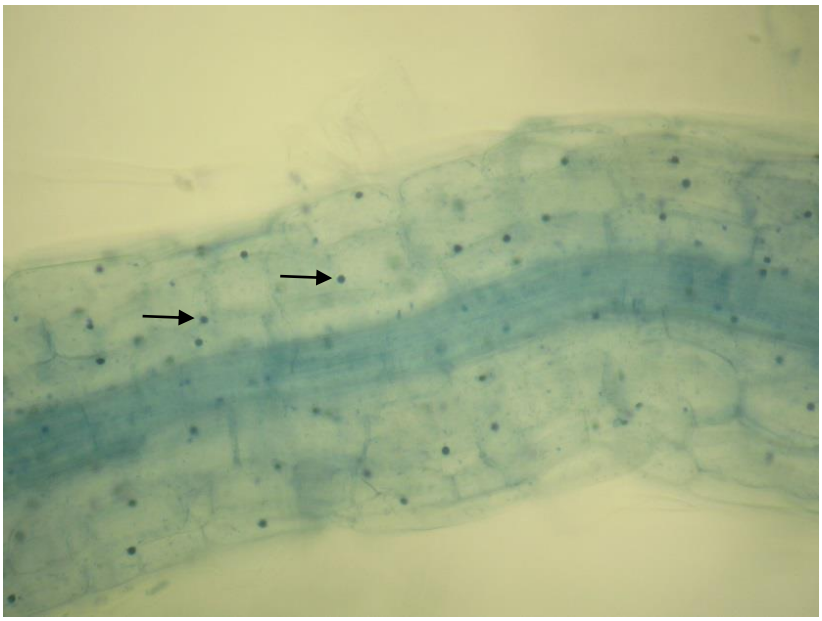

Figure 4.4. Zero P treatment saw no mycorrhizal colonisation of BG-treated grass roots, however extensive sporangia (black arrows) seen on both BG and SSI-treated roots (BG root section shown) (×200 magnification)

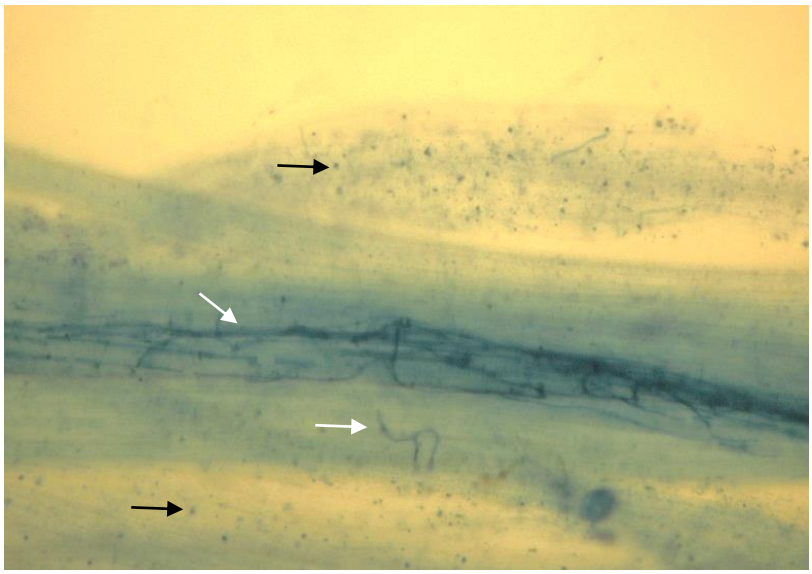

Figure 4.5. RP treatment saw increased mycorrhizal structures (white arrow) and some sporangia (black arrow) for the BG and SSI treatments (BG root section shown) (×200 magnification)

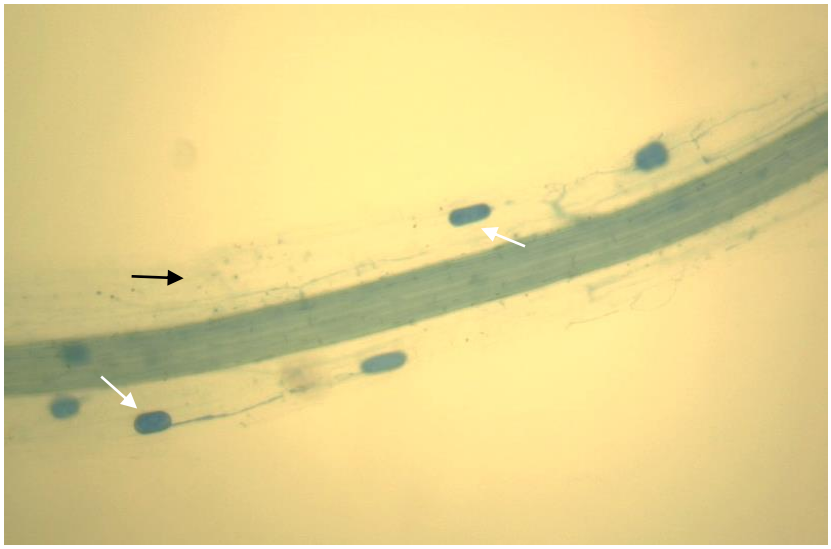

Figure 4.6. TSP application saw increased mycorrhizal structures (white arrow) with less sporangia (black arrow) for the BG and SSI treatments (BG root section shown) (×200 magnification)

4.6. Discussion

4.6.1. P-liberation

The lowering of pH and ligand exchange are widely considered to be the main mechanisms of P mobilisation of recalcitrant P_i in low P soils by plants and micro-organisms (Illmer, Schinner 1992). Dissolution of mineral (Ca) P, occurs by proton extrusion, and / or organic acid anions exchange for sorbed P (Oburger, Jones & Wenzel 2011, Zhang et al. 2011). Within this study, all BIs markedly reduced the culture medium pH (> one pH unit). These results mirror other studies in which pH was reduced by P solubilising isolates organic acid production and microbial respiration (Alam et al. 2002, Oliveira et al. 2009). P was more easily liberated from TCP than the phytate. The quantity of TCP used within the broth has been shown to be optimum at 2.5 g L^{-1} for both fungi and bacteria (Tallapragada, Seshachala 2012). It is claimed that TCP is unsuitable for P solubilisation isolation studies (Bashan, Kamnev & de-Bashan 2013). TCP has been shown to be solubilised by organisms that are not plant beneficial organisms, such as *Escherichia coli* (Kim, Jordan & Krishnan 1998). TCP, therefore, is not suitable when assessing unknown micro-organisms for potential plant beneficial P mobilisation. Within this study micro-organisms used have already been isolated based on their plant beneficial properties (Illmer, Schinner 1992, Alam et al. 2002, Mikanova, Novakova 2002, Chen et al. 2006), as such the use of TCP was deemed suitable.

Overall the study gave an indication of the potential gains both in terms of plant nutrition, but also in the potential of BIs contributing to reduce inorganic P fertiliser applications. The solubilisation study indicated that application of BIs increased plant-available P into solution by as much as 28% for PN, of the fungal based BIs, BG was found to liberate the most at 24%, BMP and SSI both 16% and 17% respectively and BS at 6%. These values are in accordance with similar studies that examined single strains of fungal and bacterial PMM's ability to increase solution PO_4 (Oliveira et al. 2009).

The mineralisation percentages of phytate were all < 5% of available P. Of the BI treatments, SSI liberated the most P at 5%. These values are much lower than those reported in other studies. MF are obligate symbionts and require a plant host to sporulate and form a hyphal network, this trial was performed without a plant host as such the total P liberating potential of the BI is likely to be higher. Hyphae of MF have been shown to both solubilise and mineralise recalcitrant P complexes (Marschner, Dell 1994, Koide, Kabir 2000). Other studies also

focused on single strains of PMM (Oliveira et al. 2009), the consortia of fungi within some of the BIs tested may have had an interactive effect which impacted on overall P-liberation. Bacteria have been shown to mobilise P from phytate (Oliveira et al. 2009), within this study the bacterial BI, PN, successfully mobilised P but only liberated ~ 2% of available PO_4 from the phytate. The initial P analysis of the broths revealed approximately 3% phosphate within the phytate, which came into solution (~30 mg l^{-1}), and was accounted for in the subsequent calculations. However, the presence of soluble P has been shown to inhibit the mineralising potential of micro-organisms (Mikanova, Novakova 2002).

Whilst mineralisation values were low, there were differences between BIs. The granular BIs, all feature fungal components, both root-associated and mycorrhizal. BG, BS and SSI all liberated more P, within the phytate broth, than BMP. BS is known to contain RAF such as *Trichoderma*. RAF are able to both solubilise P_i and mineralise P_o, e.g. *Aspergillus* and *Penicillium* spp. secreting organic acids which mobilise P_i, as well as phosphatase enzymes, allowing hydrolysis of P_o (Vassilev, Fenice & Federici 1996, Bolan 1991, Barrow, Osuna 2002). Oliveria et al. (2009) found the fungi, *Talaromyces rotundus* (basionym: *Penicillium rotundum*) the most efficient liberator of P from phytate. BMP contains no (known) root-associated fungi. The contents of BG makes no mention of RAF, but the agar plates revealed several free living fungi present within both the BG and BS, the latter also showing signs of bacterial colonies. The SSI treatment also showed signs of RAF with morphological examination suggested it being *Mortierella* sp., a known P-mobilising fungi. The SSI was a bespoke inoculant and was stated as containing one MF, *Glomus intraradices*; the presence of another fungi, albeit a plant beneficial P-mobilising fungi, does raise questions about the manufacture of commercial inoculum and problems of contamination. It is thought that the trap culture method, employed by large scale manufacturers of MF inoculum, can result in approximately 10^3 bacteria and fungi per cm^3 within BI (INVAM, personal comm.).

4.6.2. Phosphate-source bioassay

4.6.2.1. Yield, phosphorus content and PER

After six weeks growth, prior to the application of the P treatments, there were differences in yield of the BI-treated grass; BMP, BS, SSI and PN all yielding higher than the control, although not significant. Total shoot P was 2-3 fold higher for SSI and BMP, the latter significantly so. P mobilising micro-organisms featured within the BI, such as *Bacillus* spp.,

may have increased plant-available P from the very low amount of P (both plant-available and total) present within the growth media (Oliveira et al. 2009). The PER of the BS-treated grass was significantly higher than the control, the % P and total shoot P were not, suggesting that the increase in PER by BS was due to another factor, other than P. BS contains many non-living additives (Chapter 3), such as humic and fulvic acids, supplementing other plant beneficial macro- / micro-elements that could improve plant P use.

The second cut, after the application of the P treatments, saw significant yield gains for BIs, furthermore there were significant interactive effects of BI and P treatment, with some BIs yielding more with no P application, and others yielding higher with the addition of TSP. Other studies have seen similar trends, in which P mobilisation mechanisms of plant growth enhancement were dependent on both P-source and micro-organisms (Satter et al. 2008, Oliveira et al. 2009, Pagano, Scotti 2010, Naghashzadeh et al. 2013). P effect yield gains were observed within this study, for example, BS-treated grass with TSP application, and BMP-treated grass with RP application both significantly increased yield and total shoot P compared to their respective controls.

4.6.2.2. Colonisation

Micro-organisms within the BIs are known to mobilise P, within this study there were clear variations in yield and measured P parameters between BIs and P treatment, with interactive effects between treatment factors found; reflecting the varying components of each BI and the effect of available P on micro-organism function. Mycorrhizal colonisation for BS-treated grass was reduced under TSP application, the fungal component of the BI appearing to have a reduced influence on DM gains. BS contains humic and fulvic acids, which have been shown to retard the formation of occluded P and increase the water soluble P fraction (Wang, Wang & Li 1995). Previous work (Chapter 3) was able to establish that the non-living components of BS were a significant factor in increasing yield. Conversely BMP-treated grass exhibited increased MF colonisation with TSP application but reduced DM, a trend which was reversed with RP and no P treatments. The results suggest that both BS and BMP have a plant symbiotic cost dependent on P-source availability.

When no P was applied, BG, BMP and SSI-treated grass, yielded more than the control, significantly so for BG. Total shoot P was also greater than the control, significant for the SSI treatment, suggesting a P effect for SSI-treated grass. The growth media was very low in P by the second cut, as reflected in the low % P of all the treatments. However, BG-treated grass

roots showed no signs of mycorrhizal colonisation, whilst BMP-treated grass had 21% root colonisation (the highest of the three BMP P treatments) within the no P treatment. The BG-treated grass, however, did show signs of microbial activity. Microscopy revealed several structures that were likely to be RAF, such as *Trichoderma*. Sporangia of the fungi, which extensively covered the roots, saw reduced frequency with TSP compared with RP and no P treatments. The SSI-treated grass, similarly showed signs of RAF, likely *Mortierella* sp., however unlike the BG treatment, there were combinations of both mycorrhizal colonisation and RAF, for all P treatments of SSI inoculated grass, suggesting no antagonism between the fungi. Zhang et al (2011) found a positive correlation between *Mortierella* sp. and an MF (*Glomus* sp.) on mycorrhizal colonisation of *Kostelelzkya virginica*.

This study did highlight the functional diversity of MF and *Lolium perenne* with varying P availability, in which increases in colonisation from MF of individual BIs resulted in both reduced and increased yields. For example the final yield of the BS-treated grass, when no P was applied, was one of the lowest yielding BIs, with the highest fungal colonisation (> 65%), suggesting a potential cost to the plant of maintaining the fungal symbioses in a nutrient limited environment? However analysis of variance found no significant differences of MF root colonisation for BS-treated grass with different P sources. The results did show signs of high variability, and so one cannot be sure the reduced yield is correlated with the mycorrhizal colonisation rate. On the other hand, SSI-treated grass, with TSP application, had similarly high colonisation rates (> 56%) as BS-treated grass and did significantly increase yield, but as with BS, variability of the mycorrhizal colonisation rates were high and no significant differences were observed between P-treatments.

Whilst MF colonisation have been shown to be reduced with TSP application (Prasad et al. 2012, Naghashzadeh et al. 2013), BG and SSI-treated grass exhibited the opposite trend, and could be due to the inert carrier media of both BIs, which has been shown to increase root colonisation (Chapter 3) due to improved capture and retention of ions such as PO_4^{3-}, NO_3^- and NH_4^+. BS and BMP, which both saw reduced MF colonisation, with RP and TSP application, are both BIs with a bacterial fraction, as well as a fungal component. *In-vitro* studies have shown that the number of P-mobilising bacteria to be negatively correlated with soil P levels, offering the possibility that reduced bacterial numbers may lead to reduced MF colonisation (Mander et al. 2012). Using three pastures, under varying fertiliser regimes, Mander et al (2012) were able to show that *Pseudomonadaceae* and *Actinobacteria* were most strongly linked to plant-available P ($p < 0.05$). Whilst Roesti et al (2006) found a negative correlation

between MF colonisation and increasing plant-available P, and suggested the reduced MF colonisation was due to reduced abundance of P-mobilising bacteria.

BG yielded significantly higher than the control when no P treatment was applied, but MF root colonisation (F%) was zero, which, after the application of RP and TSP, increased F% (1.1% and 28.9% respectively) and, as with BS colonisation, reduced yields to the same as the control. This result is similar to other studies, which have shown reduced yield on plants colonised with MF when P limitation is removed, suggesting an MF symbiotic cost (Satter et al. 2008, Pagano, Scotti 2010, Prasad et al. 2012, Tanwar, Aggarwal & Parkash 2014, Dehariya et al. 2015). The zero P treatment yield gains were possibly due to RAF fungi present within the BI. There is some evidence within the literature to suggest a potential antagonism of RAF on MF colonisation (Summerbell 1987, Dehariya et al. 2015). The extra-matical phase of *Glomus intraradices* has shown myco-parasitism by *Trichoderma harzianum* (Rousseaeu et al 1996). Mycorrhizal colonisation in BG-treated grass however increased with the application of both RP and TSP (TSP resulting in the highest BG colonisation), a possible indication of reduced nutrient competition between the two fungi.

4.7. Conclusion

This study has demonstrated the P-liberating potential of several commercial BIs. The solubilisation and mineralisation of recalcitrant phosphate complexes was mediated by both bacterial and free living fungal components present within each BI. The study also highlighted the problems of using commercial BIs within research, potential contamination issues, and lack of information regards complete micro-organism content. As supported by other research, the MF in the BIs were variably sensitive to available soil P. This could have implications for which BI to use, and which type of micro-organism to employ within BI formulations, dependent on soil P level for example. There was a symbiotic cost to the grass for some of the BIs, when soil P level was increased, as shown by other authors. However, this was a laboratory based trial and results should be viewed with caution. Within the field, the BIs would be subject to biological, physical and chemical processes which could impact on the P-liberation mechanisms of the BIs and subsequent MF colonisation; as such future trials will examine the potential of the BIs within the field. This study has highlighted the potential of BIs to contribute

to plant-P nutrition, and could form part of an agricultural nutrient regime, contributing to reducing inorganic fertiliser inputs.

4.8. References

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CHAPTER 5: ARTICLE III

Assessing the efficacy of commercial bio-inoculum on ryegrass yields: A field trial study

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5.1. Abstract

Grasslands represent 67% of the utilised agricultural area within the UK. In the near future, greater production will be required of them to meet the demands of an increasing population. Agricultural production is reliant on the application of inorganic fertilisers to supplement plant growth with essential nutrients such as phosphorus (P). With finite supplies of rock phosphate threatening agricultural production, the use of biological inoculants is regarded a potential option to make better use of existing soil P reserves. In theory, biological inoculants could be engineered to contain micro-organisms that are able to supplement P requirements through making available soil P stored in plant unavailable forms. A field trial study was undertaken to examine the efficacy of five commercial biological inoculants (BIs) as a means to increase ryegrass yields on a typical UK agricultural soil. The soil, a brown earth Cambisol, with an initial 21 mg kg^{-1} plant-available P (a medium P soil) was seeded with a ryegrass mix (Broadsward var.). Three yield cuts over one year found significant yield gains for some of the treated grasses, but these were not due to an effect of increased phosphorus acquisition. Mycorrhizal colonisation frequency and macro- / micro-elemental analysis of the biomass revealed significant negative correlations between mycorrhizal colonisation and the uptake of elements such as Al and Fe. Overall, the study concludes that the use of BIs within soils which are not low in plant-available phosphorus may not bring significant yield gains to make them cost-effective.

5.2. Introduction

The wet, temperate climate of the UK is conducive for grass growth. Grasslands represent 67% of the UK's utilised agricultural area, and are fundamental to most livestock farming operations (Defra. 2014a). Projected rises in global population mean that demands for food, feed and fibre will require increased agricultural production (Heffer, Prud'homme 2010); placing increasing pressures on grasslands systems.

Phosphorus (P) is often a major limiting nutrient to plant growth. Increased agricultural production is inexorably linked with inorganic fertilisers use. In 2013, UK agriculture used 194 kt of mineral P fertilisers (P_2O_5); arable and grasslands receiving an average of 28 and 9 kg ha^{-1} respectively (Defra. 2014b). However, much of the P applied to agricultural land in the past is now stored in the soil as surplus P (Withers, Edwards & Foy 2001). This not only undermines current attempts to reverse the ecological damage and loss of aquatic biodiversity caused by eutrophication, but is also a potentially unutilised P resource, termed 'legacy P', that could be used to reduce applications of costly inorganic (manufactured) fertilisers, without affecting crop yields (Withers, Edwards & Foy 2001, Sattari et al. 2012, Sharpley et al. 2013). The distribution and accessibility of this essential element within the soil is extremely complex and diverse. However, establishing a reliable P supply, coupled with increased nutrient use efficiency will be essential for meeting projected agricultural demands (Lynch 2007).

In recent years there has been an increase in the use of bio-technology to improve agricultural production (Transparency Market Research 2014). Many commercial products are available that improve plant growth through a variety of mechanisms. The improved utilisation of applied mineral P fertilisers and exploitation of soil legacy P is within the remit of many of these products. Bio-inoculants (BIs) are tailored formulations utilising current understanding of microorganism function to create products for a range of soil type and cropping systems (Roesti et al. 2006, Ahmad et al. 2013). BIs contain individual, or consortia, of known microbes which have potential plant growth-promoting benefits, such as the mobilisation of legacy P. BIs contain bacterial and / or fungal micro-organisms, which utilise several mechanisms to improve nutrient availability and acquisition of P, such as mineralisation, solubilisation and translocation (Owen et al. 2015).

The success of commercial BIs should be reflected in an economic gain, either through improved yields or reduced inorganic fertiliser applications, or both. Phosphorus efficiency

ratio (PER) of BI treated plant biomass is a measure of the potential of BIs to improve nutrient acquisition through reducing plant metabolic costs, for example, reduced partitioning of P from shoots to roots; and can be assessed by the measure of yield per unit P within the forage (P-offtake) (g DM mg^{-1} P) (Hammond et al. 2009). A higher ratio is indicative of an improved conversion rate of P taken up into dry matter and reduced P removal per unit yield from the soil; thereby helping to reduce the need for inorganic P fertiliser applications.

Within the UK, > 70% of agricultural soils contain over 16 mg kg^{-1} of plant-available P (PAAG. 2014), adequate for grass growth. However, there are mixed reports regarding the effectiveness of BIs under different levels of plant-available soil P, with both negative and positive correlations reported (Schubert, Hayman 1986, Koide 1991, Grant et al. 2005, Broschat, Elliott 2009).

As well as mobilisation of P, BIs can confer other plant benefits, e.g. bacterial additions can promote plant growth promotion through hormone production, reduction of plant ethylene levels and induced systemic disease resistance. They can indirectly increase growth through bio-control mechanisms of reducing plant disease or stimulating other microbial symbioses e.g. mycorrhizas (Antoun, Prevost 2005, Martínez-Viveros et al. 2010). Fungi, such as *Trichoderma* spp. (phylum Ascomycota; order Hypocreales), can synthesise auxins which stimulate lateral plant root development (Benitez et al. 2004, Contreras-Cornejo et al. 2009) or increase plant pathogenicity (Gupta, Satyanarayana & Garg 2000). Mycorrhizal fungi (MF) are particularly important for increasing the uptake of immobile nutrients such as Zn and Cu (Clark, Zeto & Zobel 1999), or reducing the accumulation within plant biomass of elements such as Fe and Al (Shaw et al. 1990, Mendoza, Borie 1998, Lux, Cumming 2001).

There is a plethora of products on the market, all of which claim to consist of plant-beneficial micro-organisms, either as single strains or consortia; however, there is a shortage of peer-reviewed publications that report on studies using commercially available BIs, especially under robust experimental field trials (Owen et al. 2015). In this study, a field trial was established in August 2012 to explore the efficacy of three commercially-available products, within a typical UK agricultural soil, on increasing ryegrass yields.

5.3. Aims

- To test the effects of three commercially-available soil BIs on the yield of a grass ley mix in a typical UK agricultural soil

- To measure changes in fungal root colonisation with the application of BIs

- To ascertain improvements in the PER of grass inoculated with BIs

5.4. Methodology

5.4.1. Site

A field trial was established at Henfaes Research Centre, Abergwyngregyn, UK (53°14′ N, 4°01′ W) (Fig. 5.1), which is characterized as having a temperate-oceanic climate regime. The soil, a Eutric Cambisol, developed since the last glacial period (10,000 ybp). The main soil chemical characteristics were analysed by Glenside (Livingston, UK) and NRM (Berkshire, UK), and are presented in Table 5.1. Plant-available P was measured at 21 mg kg^{-1}, and a short preliminary test prior to the study did find BI-treated grass yielded more than non-treated grass (Glenside, oral comm.), however the trial did not feature replicates and as such the result was not tested statistically.

Table 5.1. Soil characteristics, pH, cation exchange capacity (CEC), organic matter (OM) and elemental content of the soil (mg kg^{-1}) (Glenside Albrecht ®, Livingston, UK. ISO/IEC 17025-2005). Nitrate (NO_3) and ammonium (NH_4), with total and plant-available phosphorus (P) and total potassium (K) (NRM Ltd., Berkshire, UK). Values are of the entire field site

			N*		**P**		**K**
pH	**CEC (meq 100 g^{-1})**	**OM (% w/w, LOI**)**	**NO_3-N (mg kg^{-1})**	**NH_4-N (mg kg^{-1})**	**Total (mg kg^{-1})**	**PO_4-P^x (mg kg^{-1})**	**Total^y (mg kg^{-1})**
5.5	14.65	5.6	12	23	1199	21	862

Elemental content (mg kg^{-1})									
Ca	**Mg^z**	**Na**	**Fe**	**Mn**	**Cu**	**Zn**	**Cl**	**I**	**Mo**
3359	254	125	827	184	6.4	18.9	52	0.09	2.24

**loss on ignition
*Total N measured at 0.28% (w/w), of which 139 kg ha^{-1} N available (30 cm profile)
^{x}agricultural P index of 2
^{y}agricultural K index of 2+
^{z}agricultural Mg index of 2

The site has a mean annual temperature of 9.8 °C, and mean annual rainfall of 800 mm (Farrell et al. 2011). The site, marked out in figure 5.1, was a long term ley (25 years), originally seeded with *Lolium perenne* and *Trifolium repens* (60 : 40); with an annual split dose application of 120 kg ha^{-1} N and 20 kg ha^{-1} P. The original sown cultivars had worn out naturally, and secondary grasses such as *Agrostis stolonifera*, *Holcus lanatus* and *Poa annua* dominated the sward. The site was re-seeded in August 2012, at 34 kg ha^{-1} (Oliver Seeds, UK) using a seed

spreader. Grass seed used was the variety Broadsward (Oliver Seeds, UK); a mix of perennial and hybrid ryegrasses (*Lolium* spp.).

Figure 5.1. Location of field trial at Henfaes Research Centre (Abergwyngregyn) (Imagery ©2012, Infoterra Ltd & COWI A/S, DigitalGlobe, GeoEye, Getmapping plc, Map data ©2012 Google)

5.4.2. Treatments

Prior to application, BIs were combined with 10 g of sterile vermiculite to help with distribution due to small volumes used. BI treatments were applied by hand to a central treatment area (1 m^2) within the plot area of 4 m^2, to avoid edge effects (Appendix 9.6.1). Control plots received 10 g of vermiculite only. The BIs applied were at manufacturer's recommended rates, and ten times the application rates. Table 5.2 gives a brief description of each BI. Elemental analysis revealed that BI application would result in only a very low input of nutrients (see Chapter 3, Section 3.5.1.1), therefore no sterilised controls were necessary. No fertilisers were applied at seeding (August 2012), to prevent any deleterious effects of inorganic fertilisers on fungi contained within the BI treatments (Veverka, Stolcova & Ruzek 2007). Eight weeks after seeding, an establishment cut was taken (October 2012). Fifty kg ha^{-1} of N (34.4% N, ammonium nitrate) was applied in June 2013, six weeks before the first cut was taken in July 2013. A further 50 kg ha^{-1} of N was applied after the first cut. The second yield cut was taken in September 2013. Fertiliser application rates for nitrogen were slightly below the RB209

recommendations of 60-70 kg ha^{-1} but any more may have had detrimental effects on fungi within the soil (Veverka, Stolcova & Ruzek 2007), which may have affected later analysis of fungal soil communities.

The site was laid out in a randomised strip design (Fig. 5.2), with each BI treatment replicated within each application rate row (recommended and ten times) (n = 3). Rows were separated by a two meter buffer strip, to which no fertilisers were applied.

Table 5.2. Composition and recommended application rates of commercial bio-inoculants (BI)

BI*	**Composition**		**Rec. app. rate (kg ha^{-1})**
Biagro® Grass (BG)	Granule	Consortium of five MF within a vermiculite carrier < 0.5% additives	1
Biagro® MP (BMP)	Powder	Consortium of MF and phosphate mobilising bacteria	0.150
Biagro® S (BS)	Powder	Consortium of MF and bacteria with humates, algal extracts and amino acids	0.125

*supplied by Glenside Group (Livingston, UK)

5.4.3. Biomass measurements

After each harvest, plant samples were randomly taken from the central treatment area (1 m^2) (Appendix 9.6.1), and separated into shoot and root, shoots were weighed and dried in an oven (80 °C) for 24 hours to calculate dry matter yield (DMY). The DM was then dry-ashed (550 °C / 16 h) and the residue dissolved in 0.5 M HCl. The total P content of the forage was then determined using the ascorbate / molybdate blue method (Murphy, Riley 1962). Roots were examined for fungal colonisation; briefly, roots were rinsed, cleared using KCl and stained with Trypan blue (Phillips, Hayman 1970) and colonisation quantified using Mycocalc (Trouvelot, Kough & Gianinazzi-Pearson 1986) (see Chapter 3, Section 3.4.6-7 for full description). Subsamples of forage were sent to Sciantec (UK) for elemental analysis (Al, Ca, Co, Cu, Fe, I, K, Mg, Mn, Mo, Na, S and Zn) (first cut only).

5.4.4. Statistical analysis

Data were analysed for statistical significance with SPSS 22.0 (IBM). Percentage data was log transformed. All data were analysed for normality using Shapiro-Wilk test. Analysis of variance (Anova) identified any significant results within each factor, post hoc, Fisher's LSD, tested for any significant individual treatment effects. Results were considered to be significant at the $p < 0.05$ level. All data was suitably transformed, if required, to conform with Levene's test of homogeneity. Pearson's r analysis was used to explore correlations; the statistic was based on 1000 stratified (BI treatment) bootstrap samples, with bias control accelerated 95% confidence intervals.

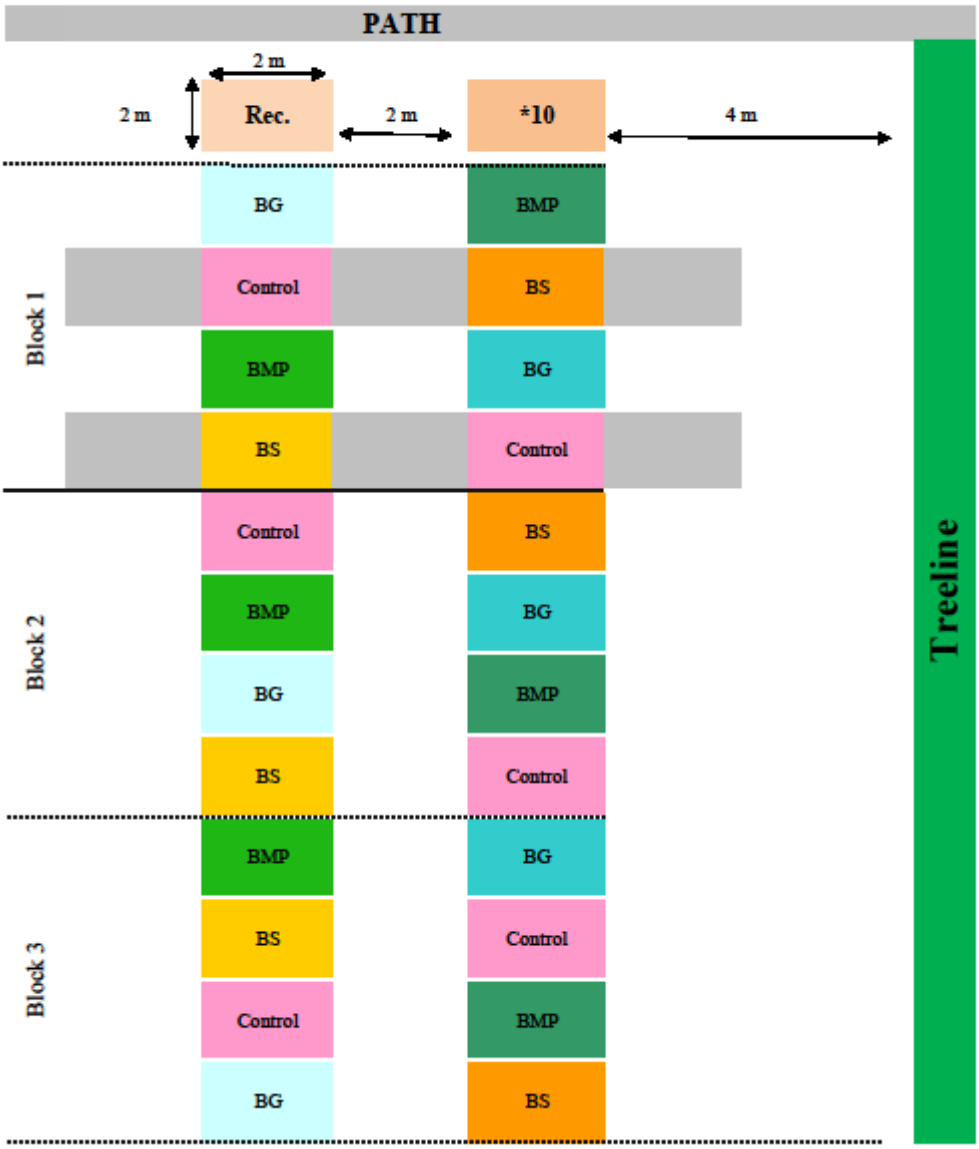


Figure 5.2. Plot layout of Gadlas field trial. Plots were 2 m^2 and separated by a 2 m buffer strip. Sampling was taken from a central 1 m^2 area within each plot. Grey plots had no fertiliser or treatment applications. Each strip consisted of three blocks of three BI treatments (Biagro Grass (BG), Biagro MP (BMP), Biagro S (BS)) and a control, a total of 12 plots per application rate (recommended (Rec) and ten times recommended (*10))

5.5. Results

5.5.1. Yield and total forage P

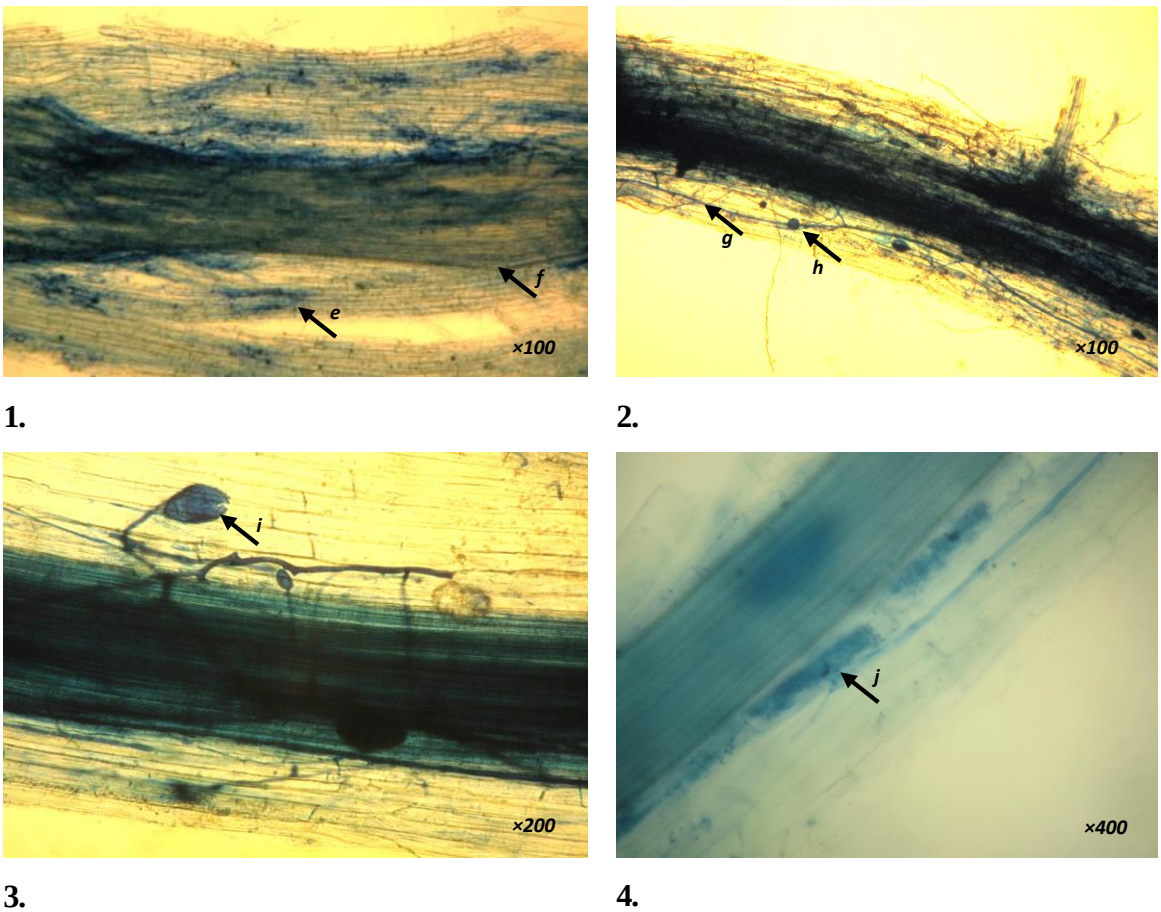
Results across all three cuts (establishment, 1st and 2nd) did not reveal any significant treatment or concentration effects on yield and total forage P (Appendix 9.6.3). Examining individual cuts, however, did reveal significant treatment effects (Table 5.3). For example in the first cut, BG-treated grass yielded higher than all other treatments at both recommended and ten times application rates, the former significant (Anova, $p < 0.05$) (Table 5.3). Non-significant differences in % P and total forage P would suggest yield gains by BIs were not due to a P effect. There were no significant differences of BI treatment, or application rate, for any of the measured parameters in the second cut (Appendix 9.6.2). Both fungal colonisation parameters, however, significantly increased compared with the first cut, for all treatments, including the control plots, at both application rates (F% > 88%) (Appendix 9.6.2).

Table 5.3. First cut, mycorrhizal frequency of colonisation (F%), intensity of colonisation (M%), dry matter yield (DMY) (t ha^{-1}), % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P), values in parenthesis are ± 1 standard deviation. Different superscript letters represent significant differences between BIs within application rate ($n = 3$) (Anova, LSD post hoc, $p < 0.05$)

Application rate	Treatment	DMY (t ha^{-1})	F %	M %	% P	Total forage P (kg ha^{-1})	PER (g DM mg^{-1} P)
Recommended	*Control*	3.84[a] (± 0.35)	53.3 (± 14.5)	10.4 (± 4.6)	0.32 (± 0.02)	12.24 (± 0.48)	0.31 (± 0.02)
	BG	5.00[b] (± 0.70)	61.1 (± 22.2)	10.4 (± 0.7)	0.26 (± 0.05)	12.60 (± 1.12)	0.40 (± 0.09)
	BS	3.66[a] (± 0.35)	46.5 (± 7.9)	8.9 (± 5.8)	0.31 (± 0.02)	11.32 (± 0.99)	0.32 (± 0.02)
	BMP	3.72[a] (± 0.27)	37.8 (± 12.6)	5.3 (± 4.1)	0.30 (± 0.03)	11.10 (± 0.31)	0.34 (± 0.03)
×10	*Control*	3.37 (± 0.73)	23.3[a] (± 11.5)	4.7 (± 4.1)	0.31 (± 0.04)	10.16 (± 1.32)	0.33 (± 0.05)
	BG	3.88 (± 0.17)	48.9[b] (± 6.9)	9.8 (± 4.2)	0.30 (± 0.03)	11.78 (± 1.49)	0.33 (± 0.03)
	BS	3.53 (± 0.39)	28.1[a] (± 5.0)	4.9 (± 3.0)	0.32 (± 0.02)	11.37 (± 0.77)	0.31 (± 0.01)
	BMP	3.66 (± 0.42)	21.5[a] (± 12.3)	5.3 (± 6.0)	0.32 (± 0.02)	11.72 (± 1.52)	0.31 (± 0.02)

5.5.2. Colonisation

The first and second cut grass roots were stained and analysed for fungal activity. All roots examined, across all treatments including the control, were colonised by fungi; many fungal structures were visualised (Fig. 5.3.1-4). BG-treated grass exhibited higher fungal colonisation rates (F%) at both application rates, significant for the ten times rate (Anova, $p < 0.05$) (Table 5.3). The overall mean (total of cuts one and two) F% and M% for all BI treatments were found not to differ significantly from controls (Appendix 9.6.3). Staining of root sections revealed several fungal structures known to play a role in mediating plant-P uptake. Hyphae along the root cortex (Fig. 5.3.2) support the bi-directional transfer of plant-beneficial nutrients, such as N and P, to the host plant and photosynthate carbon to the fungi. Arbuscules are areas of nutrient exchange between the plant and fungal symbionts (Fig. 5.3.4).



1. **2.**

3. **4.**

Figure 5.3.1. (*e*) fungal structures within the root cortex appear as light blue; (*f*) root stele is a darker blue **2.** (*g*) extensive root colonisation, with the appearance of hyphae; (*h*) vesicles, storage structures **3.** (*i*) chlamydospore **4.** (*j*) arbuscule, plant-fungal nutrient interface

Correlation analysis of total F% and M% of all treatments (including controls), with the DMY, % P and total forage P and PER, showed some significant correlations (Table 5.4), indicating the role of fungi in mediating P uptake. Significant interactions were observed for F%, with DMY ($p = 0.037$) % P ($p = 0.019$) and total P uptake ($p < 0.01$), all positively correlated, whilst being negatively correlated with PER.

Table 5.4. Pearson's correlation coefficients of mycorrhizal F%, M% with DMY, % P, total forage P and PER. Statistic based on 1000 bootstrapped stratified samples with bias correction

Total[z]	DMY	% P	Total forage P	PER
F%	0.306*	0.340*	0.454**	-0.216
M%	0.101	0.020	0.135	-0.030

[z] cut 1 and 2
*. Correlation is significant at the 0.05 level (2-tailed)
**. Correlation is significant at the 0.01 level (2-tailed)

5.5.3. Macro- / micro-elements

Elemental analysis of the forage was conducted for the first cut only. There were no significant effects of BI application on any of the measured elements (Appendix 9.6.4). However, as with % P, forage P and PER (Section 5.5.2), there were significant correlations of MF colonisation (F%) and various elements. For example, there were significant negative correlations of Fe and Al content with increased mycorrhizal colonisation (Table 5.5).

Table 5.5. Pearson's correlation coefficients of mycorrhizal frequency (F%) with Fe and Al (mg kg^{-1}) of first cut. Statistic based on 1000 stratified samples with bias correction (BCa) showing 95% confidence intervals (CI), outliers removed ($n = 20$)

			Fe	Al
Correlation Coefficient			-.719**	-.606*
Sig. (2-tailed)			<.0001	.005
N			20	20
Bootstrap[c]	Bias		.011	-.013
	Std. Error		.212	.195
	BCa 95% CI	Lower	-.871	-.800
		Upper	-.466	-.404

*. Correlation is significant at the 0.05 level (2-tailed)
**. Correlation is significant at the 0.01 level (2-tailed)

5.6. Discussion

Of the limited studies which have examined the efficacy of commercial BIs within the lab and field, results are varied, inconsistent and contradictory (Owen et al. 2015). There is no consensus on the efficacy of BI products. The efficacy of any fungal or bacterial strain utilised within inoculants is subject to numerous soil, crop and environmental factors, from crop species compatibility, size and effectiveness of indigenous microbial populations, soil fertility and management (Bashan et al. 1995, Adholeya, Tiwari & Singh 2005). Priority effects, in which initial populations of species determine final community composition of the plant rhizosphere, will also have an impact (Mummey, Antunes & Rillig 2009, Verbruggen et al. 2013). Collectively, these affect the soil microbial dynamics, functional processes and performance of any applied commercial BIs.

One of the main marketable benefits given for using commercial BIs, is their potential to enhance P acquisition and uptake, contributing to reducing the need for costly inorganic fertilisers. BI products are primarily targeted at low P soils ($P < 15$ mg kg^{-1}), though within this study, soil P prior to seeding was measured at 21 mg kg^{-1}, rendering the use of BIs, with respect to P, potentially unnecessary. For all harvest cuts, there were no significant differences found for any of the measured P parameters, suggesting any yield gains were not due to a BI-mediated P effect. There were significant yield gains within individual cuts for some BI-treated grasses; for example, BG-treated grass, at recommended application rates, yielded significantly higher than all other treatments for the first cut. The proceeding yield cut, however, showed no significant effect of BI application. Stamenov (2012) investigated the use of individual strains of plant growth-promoting fungi and bacteria on yield gains of *Lolium perenne*. The authors found that, of the tested micro-organisms, *Pseudemonas fluorescens* (phylum Proteobacteria; order Pseudomonadales) was the only organism to significantly increase yield in the first cut, but also failed to significantly increase yields with subsequent cuts. Interestingly, the authors also observed a lag effect of tested organisms in which a *Streptomyces* sp. (phylum Actinobacteria; order Actinomycetales) increased yield significantly by the second cut, although this too did not continue into a following cut (Stamenov et al. 2012). The lack of a significant yield in proceeding cuts would suggest either a possible problem of persistence of the applied micro-organism within the soil, or that native populations of micro-organisms had

re-established and were providing similar plant benefits as the BI treatments. For example, tillage has been shown to disrupt fungal mycelial networks (Maherali, Klironomos 2007).

There were statistical differences between cuts for MF colonisation frequency; total mean colonisation frequency of grass roots was 38% in the first cut compared with 91% in the second cut. The first cut was taken nine months after seeding, to allow adequate time for grass establishment over winter and into the spring. This gave ample time to allow colonisation of the root by the MF component of the BIs. MF require approximately 12-16 weeks to fully colonise roots (INVAM, personal comm.). BG-treated grass exhibited the highest frequency of MF root activity, at both application rates, and was significant for the ten times application rate. By the second cut, however, there were no significant differences of MF colonisation for any of the applied BIs. This could be due to numerous factors such as seasonality, which has been shown to affect MF occurrence (Sanders, Fitter 1992, Smilauer 2001), or BI persistence within the soil, in which introduced species have been shown to decline over time (Bashan et al. 1995). There could be other factors, such as increased competition from the re-establishment of native MF. For example priority effects, in which the initial disturbance of native fungal mycelial networks may have allowed introduced fungal populations to colonise roots more easily (Mummey, Antunes & Rillig 2009, Verbruggen et al. 2013). BG was found to contain *Trichoderma* sp. (Section 7.5.1), some strains are known to be particularly aggressive rhizosphere competent fungi (Harman 2000), and have the potential to have negative effects on MF colonisation and persistence within the rhizosphere (Brimner, Boland 2003). This is attributed to the inherent capacities of some *Trichoderma* sp. to control pathogenic organisms, particularly fungal pathogens, and hence the potential to suppress and out-compete MF for essential resources (Harman 2000, Brimner, Boland 2003).

Tilling also greatly influences spatial aggregation, and thereby competitive interactions between micro-organisms (Bever et al. 2009, Verbruggen et al. 2012). Commercial BIs generally contain mycorrhizal species from the *Glomus* genera, which have been shown to be better adapted to soil disturbance than others, such as *Gigaspora*, *Scutellospora* and *Racocetra* (Maherali, Klironomos 2007). *Gigaspora* and *Scutellospora* have been shown to form fewer intramycelial anastomoses (hyphal fusions) than *Glomus* (De La Providencia et al. 2005) and hence might be more susceptible to disturbance of established mycelial networks.

BG-treated grass roots of the first cut were found to have the highest mycorrhizal root colonisation (F%) at both application rates, 48.9% (ten times) and 61.1% (recommended),

significant for the former. However, the experimental design of the field trial saw a statistically significant difference between the total mean F% of all samples within each strip (including the control); in which the first cut saw a mean total F% of 46.5% for the recommended strip and 30.5% for the ten times strip ($p < 0.05$). This would suggest a gradient between the two treatment rows, in which soil properties, nutrient content for example, impacted on MF establishment and subsequent root colonisation, as such claims of increased root colonisation with increased application of BI are not robust.

Nitrogen has been shown to play a role in MF establishment, with NH_4-N shown to be positively correlated with MF colonisation rates (Ali et al. 2009, Leigh, Fitter & Hodge 2011). Soils from each application rate treatment strip were analysed for N, P and K, and levels of NH_4-N and NO_3-N were found to differ between application rows within the field (Appendix 9.6.5). NH_4-N was found to be almost twice as high within the recommended treatment row and may account for the difference between treatment row colonisation rates.

The % P content of the BI-treated grass and total P uptake were both found to be non-significant from controls, suggesting that BI-mediated yield gains were due to mechanisms other than enhanced P nutrition. However, within the field, it was not possible to prevent mycorrhizal colonisation of control plots and treatment plots by native MF; therefore, P-mediated benefits of applied BIs may not be as apparent as they would be, compared to the more sterile and controlled laboratory studies.

The elemental content of the biomass was also found to be non-significant between BI-treated grass and controls. Introduced fungal species may not be acclimatised to specific soil conditions, for example varying plant-nutrient availability (Khan et al. 2009). Significant plant / MF co-adaptation to local nutrient conditions has been shown (Johnson et al. 2010, Antunes et al. 2012), for example, the native MF (*Funneliformis mosseae*) of a mine waste soil increased transfer of arsenic from root to shoot in *Plantago lanceolata*, whilst a non-native strain of the same MF restricted plant absorption (Enkhtuya, Rydlová & Vosátka 2000). However, within this study, the MF colonisationof all the treatments, including the controls, exhibited high levels of MF colonisation by the final cut (Appendix 9.6.2) and, as already mentioned, it was not possible to differentiate between native and applied MF within the root colonisation analysis of BI-treated grass, therefore it is possible that applied BI fungi were displaced by native MF by the end of the study.

The results were able to highlight some positive plant benefits of MF colonisation. Correlation analysis revealed a moderate, but significant, positive correlation of mycorrhizal colonisation on yield and the amount of P within the biomass (% P and total forage P (kg ha^{-1})) ($p < 0.001$). MF colonisation was also significantly correlated with the several micro-elements within the biomass, notably Fe and Al. Fungi and associated bacteria have been shown to supply other macro- / micro-nutrients, and confer additive benefits to host plants by reducing the uptake and accumulation of toxic elements such as Al (Mendoza, Borie 1998, Lux, Cumming 2001), in which MF mediate interactions between Al, P_i and roots (Marschner, Dell 1994). Mycorrhizal hyphae sequester Fe and Al and prevent transport to plant (Shaw et al. 1990, Chatzistathis et al. 2013). Whilst BI application was found to not significantly effect the elemental content, these results further support the benefits of MF in general to plant growth.

5.7. Conclusion

Within this study, BI mediated yield gains were found to be limited, in which some of the tested BIs increased yields of ryegrass over the control for some cuts; and the moderate yield gains were not as a consequence of increased plant-P uptake and use. However, after three cuts, overall total BI mediated grass yields did not differ to that of the controls. Suggesting earlier disruption of the soil, during tillage for example, allowed applied BIs to successfully colonise roots but these effects were relatively short lived.

Of the tested BIs, BG was the only one to exhibit increased mycorrhizal colonisation, as with the yield howvere increases were modest and restricted to one cut. Furthermore, it was not possible to conclude that BI application increased mycorrhizal colonisation due to high variability between treatments. This highlights a particular problem of investigating potential positive benefits of BIs at a field scale; i.e. native populations, heterogeneous nutrient distribution, and fluctuating environmental conditions all compound uncertainty when trying to account for positive treatment effects.

The study did highlight the significant contribution of the mycorrhizal symbioses to plant-P nutrition, and the significant contribution of MF to grass yield. The uptake of more harmful elements such as Al was found to be negatively correlated with mycorrhizal colonisation

further emphasising the important role mycorrhizae and other root-associated micro-organisms have within the plant root rhizosphere.

Whilst BIs can increase yield through improved P nutrition, early yield gains within this study, were more likely due to increased N availability after tilling. BIs can improve grass yields; however, the application rates of this study (manufacturer's recommended rates, plus ten times those rates) were probably insufficient to maintain a significant persistence within the soil. The results of this study would suggest that the use of BIs within soils which are not nutrient-limited has insufficient yield gains to make them cost-effective.

5.8. References

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CHAPTER 6: ARTICLE IV

Investigating the interactive effects of phosphate and commercial bio-inoculants on the yield of ryegrass: A field trial study

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6.1. Abstract

In recent years there has been an increase in the development, and use, of biological inoculants (BIs). BIs have been shown to reduce inorganic fertiliser inputs and could be a fundamnetal component of an agricultural nutrient management program. Micro-organisms contained within BIs have been shown to increase plant availability of beneficial nutrients, such as phosphorus (P) and nitrogen (N), however positive lab-based trial results of BIs are often not replicated when applied at the field scale. Two field trials were undertaken in 2013 / 14 at Henfaes Research Centre, Abergwyngregyn, UK. One explored the effects of increasing plant-available P on the effectiveness of a BI (Biagro® Grass (BG)) on *Lolium perenne* growth. The second trial was established to examine the interactive effects of P-fertilisers, triple super phosphate (TSP) and rock phosphate (RP), and BIs; effectiveness within the field of single and consortia BIs and overall yield gains from BI treated grass on a low P soil (8 mg kg^{-1}). Three commercial inocula were selected for the trial, a consortia of mycorrhizal fungi (MF) (Biagro® Grass (BG)), a single species MF inoculant (SSI) and a bacterial suspension (Biagro® PhosN (PN)).

Results of the first trial found a positive correlation of plant-available P with plant biomass P of BG-treated grass, however, did not result in any significant yield gains. The increased P acquisition and lack of dry matter yield would suggest that yield limitation was not driven by P. Results of the second field trial found no significant interactive effects between P-fertiliser treatments and BIs; although there were significant yield gains, in which both SSI- and PN-treated grass yielded significantly more than controls. With no significant BI-treatment effects on the % P and total forage P, yield gains were due to other factors other than P. All BI treatments increased N content of the forage, across the three harvests, the bacterial suspension significantly increased forage N of the final harvest. The second field trial also found that the single species MF inoculant yielded more than the consortia. Suggesting variations between single and consortia MF products, and that the functional redundancy offered within consortia BIs may not be as effective as a singly introduced mycorrhizal fungi.

Overall, this study was able to show that the application of MF bio-inoculum can increase dry matter yields of *Lolium perenne* on P-limited soils. However, the yield increases may be more due to improved uptake of nitrogen and micro-nutrients other than P. Bacterial BI was shown to alleviate N limitation, and could potentially form part of a dual application of MF and bacterial suspension.

6.2. Introduction

An increasing global population requiring food, feed and fibre has placed increased pressures on finite supplies of inorganic fertilises (Cordell, White 2011, Van Kauwenbergh 2010, Cordell, Drangert & White 2009). This has led to an increase in the development, and use, of biological inoculants (BIs) (Transparency Market Research 2014). BIs have been shown to reduce inorganic fertiliser inputs (Blal et al. 1990, Vessey 2003, Salimpour et al. 2010, Prasad et al. 2012) and can be an integral component within an integrated nutrient management program. However, the efficacy of BIs, and their ability to provide benefits within a range of biotic and abiotic environments has been questioned (Schenck zu Schweinsberg-Mickan, Müller 2009, Owen et al. 2015).

Commercial BI formulations can contain individual strains, or consortia, of plant growth-promoting micro-organisms, both fungal and bacterial (Roesti et al. 2006, Ahmad et al. 2013) which are added to soil, or as a seed coating when re-seeding. The benefits of BI application include abiotic stress alleviation, and improved soil aggregation, to the enhancement of, both macro and micro, elemental plant beneficial nutrients such as phosphorous (P) (Azcon-Aguilar, Barea 1996, Barea, Azcon & Azcon-Aguilar 2002, Rillig, Mummey 2006). Those microorganisms with specific attributes for mobilising P are termed phosphorus-mobilising microorganisms (PMM) (Owen et al. 2015). Mobilisation of P is mediated by PMM through several mechanisms, including solubilisation, mineralisation and translocation. However, there have been many factors identified which can affect BI efficacy, such as soil clay, nitrogen and organic matter content and soil P, (Bashan et al. 1995, Oehl et al. 2010, Marschner, Crowley & Rengel 2011, Wagg et al. 2011, Yousefi et al. 2011).

Within soil, plant-available P concentrations are inherently low (Marschner 1995) and any applied P, in the form of both inorganic and organic fertilisers, can become unavailable to plants through processes of fixation. Reduced plant soluble phosphate complexes are formed when, for example, hydrous iron, aluminium oxides and luminosilicates, a feature of acidic soils, react with phosphate solutions to produce iron and aluminium phosphates, whereas calcareous soils promote the precipitation of calcium phosphates (Stevenson, Cole 1999). There are mixed reports regards BI effectiveness and the degree of plant-available soil P, with both negative and positive correlations reported (Schubert, Hayman 1986, Koide 1991, Grant et al. 2005, Broschat, Elliott 2009).

The P index is a method of quantifying the amount of plant-available P within a soil. The index represents a range of plant-available soil P that can be used by farmers to adjust conditions to favour a particular crop through the application of inorganic or organic fertilisers. The index represents a mean measurement of an entire site which, depending on the sampling technique and resolution of sampling, may not adequately reflect the heterogeneous nature of P within the soil. The tortuosity of soil and the immobile nature of P could lead to areas of higher and lower P which could impact on BI effectiveness.

The use of triple super phosphate (TSP) to supplement plant-P requirements is common in commercial agriculture, although not very efficient in calcareous and alkaline soils (Mclaughlin, Alston & Martin 1988). After application, soil solution P moves into less plant-available, non-labile, pools (Yang et al. 2012). Of applied fertiliser, the water-soluble fraction that remains within the soil solution is relatively small, at around 13% (Johnston, Dawson 2005). Rock phosphate (RP) offers a cheaper alternative to TSP, but is not suitable for direct use in agriculture because of its low solubility. Combined application of PMM, however, can increase RP solubility and subsequent plant availability (Blal et al. 1990, Singh, Kapoor 1999, Vessey 2003, Salimpour et al. 2010, Prasad et al. 2012). For example, Singh (1999) increased wheat yields by 40% when RP was applied in combination with PMM.

BI design frequently utilises many genera of microorganisms, offering functional redundancy and / or added plant benefits other than increased P supply. The majority of the non-commercial literature concerning the beneficial effects of PMM focuses on individual microbial species or strains (Vessey 2003, Aseri et al. 2008, Prasad et al. 2012). Single species PMM can have beneficial effects (Roesti et al. 2006, Jansa, Smith & Smith 2008), but mixed inocula have been shown to be more flexible and productive within variable abiotic and biotic environments (Hart, Forsythe 2012, Malusá, Sas-Paszt & Ciesielska 2012), conferring additive benefits such as increased macro- / micro-elemental uptake and improved pathogen resistance, for example (Oehl et al. 2001, Maherali, Klironomos 2007, Sikes, Powell & Rillig 2010). Conversely, dual inoculation has often been shown to have no effect, or even a negative effect on plant growth (Felici et al. 2008). For instance, positive growth responses have been reversed by dual inoculation, even though plant root colonisation rates of the multi-inoculants remained the same (Dodd, Ruiz-Lozano 2012).

Several mechanisms mediate microbial antagonism, including growth inhibition by diffusible antibiotics and volatile organic compounds, to competition for colonisation sites / nutrients /

minerals (Berg 2009). The mycorrhizal fungi (MF), *Glomus* spp., used in most commercial BIs, have been shown to have many PMM antagonists e.g. *Aspergillus niger*, *Fusarium solani*, S*treptomyces* spp., *Trichoderma harzianum* and *T. koningii* (Edwards, Young & Fitter 1998), and *Scutellospora* spp. (Jeong, Lee & Eom 2006). The root-associated fungi, *Trichoderma*, is a known myco-fungicide (as well as a PMM) and could potentially inhibit MF within mixed formulations (see Kaewchai, Soytong & Hyde 2009 for full antibiosis review of *Trichoderma*). Due to commercial sensitivity in disclosing blend formulations, investigating such effects is difficult with commercially available BIs.

Positive lab-based trial results of BIs are often not replicated when applied at the field scale. As mentioned, there are many confounding factors that mask PMM effects under suboptimal field conditions. For example, much of the experimental data using MF inocula use single host plants and single strains of fungi. Under natural conditions, the initial carbon cost of fungal symbioses (which can be as high as 20% of plant photosynthate C (Smith, Read 2008)) to a seedling would be greatly reduced if the MF colonising the roots were part of a pre-existing common mycelial network connected to adjacent established plants (Hodge, Helgason & Fitter 2010). Also, the sterile experimental conditions may impact MF colonisation strategies, due to the lack of ubiquitous soil bacteria which are known to affect MF colonisation rates (Dhillion 1992).

A field trial was undertaken in July 2013 to explore the interactive effects of P-fertilisers, TSP and RP, and BIs on yields of *Lolium perenne*. Three commercial inocula were selected for the trials, a consortia of MF (Biagro® Grass), a single species inoculant (SSI) and a bacterial suspension (Biagro® PhosN). The site also offered a unique opportunity to explore the effects of increasing plant-available P on the effectiveness of a BI, and a second trial was established to examine the use of Biagro® Grass on plots of varying P index.

6.3. Aims

- To test the effects of three commercially available soil bio-inoculants on the yield of a grass ley mix in a low P soil

- To examine the effects of an increasing soil P index on the effectiveness of BI used in a grass ley

- To test the interactions of two inorganic P fertilisers and three commercial BIs on grass growth

- To investigate the effects of a single species BI against a consortia of plant growth-promoting species on grass yields

6.4. Methodology

6.4.1. Site

A field trial was established at Henfaes Research Centre, Abergwyngregyn, UK (53°14′ N, 4°01′ W) (Fig. 6.1), which is characterized as having a temperate-oceanic climate regime. The soil, a Eutric Cambisol, developed since the last glacial period (10,000 ybp). The site has a mean annual temperature of 9.8 °C, and mean annual rainfall of 800 mm (Farrell et al. 2011).

Figure 6.1. Location of field trial at Henfaes Research Centre (Abergwyngregyn) (Imagery ©2012, Infoterra Ltd & COWI A/S, DigitalGlobe, GeoEye, Getmapping plc, Map data ©2012 Google)

Previous site history included *Lolium perenne* with *Trifolium repens* and *Cichorium intybus* (2010), followed by potato (*Solanum* var.; 2011) and barley (*Hordeum vulgare* L.; 2012). The main soil chemical characteristics were analysed by Glenside (Livingston, UK) and NRM (Berkshire, UK), and are presented in Table 6.1.

Table 6.1. Soil characteristics, pH, cation exchange capacity (CEC), organic matter (OM) and elemental content of the soil (mg kg^{-1}) (Glenside Albrecht ®, Livingston, UK. ISO/IEC 17025-2005). Nitrate (NO_3) and ammonium (NH_4), with total and plant-available phosphorus (P) and total potassium (K) (NRM Ltd., Berkshire, UK)

			N*		P		K
pH	**CEC (meq 100 g^{-1})**	**OM (% w/w, LOI**)**	**NO_3-N (mg kg^{-1})**	**NH_4-N (mg kg^{-1})**	**Total (mg kg^{-1})**	**PO_4-P[x] (mg kg^{-1})**	**Total[y] (mg kg^{-1})**
5.8	7.66	2.8	5.3	6.2	500	12	595

Elemental content (mg kg^{-1})

Ca	Mg[z]	Na	Fe	Mn	Cu	Zn	Cl	I	Mo
2088	123	77	1093	152	4.3	9.2	28	0.15	1.06

**loss on ignition
*Total N measured at 0.26% (w/w), of which 46 kg ha^{-1} N available (30 cm profile)
[x]agricultural P index of 1
[y]agricultural K index of 2-
[z]agricultural index of 2

Soil characteristics presented in Table 6.1 are based on 30 samples taken across the whole field site (396 m^2) using the widely used 'W' soil sampling technique. Soil analysis of plant-available P showed the site to be of an agricultural P index of 1 (9-15 mg kg^{-1} PO_4-P). However, within soil, P is very immobile with a very slow rate diffusion rate, orthophosphate ions move approximately 10^{-12} – 10^{-15} m s^{-1} (Marschner 1995); therefore, the distribution of plant-available P is not always homogenous. The W sampling technique fails to take into account the heterogeneous distribution of P at smaller scales, leading to the failure to detect potential "hotspots" of P. The site was divided into 2 m^2 plots (99 in total). Each plot was measured, from a bulk sample of five soil cores, for pH, EC (µS cm^{-1}) and PO_4-P. The plant-available soil phosphate fraction was measured using the Olsen, sodium bicarbonate, extraction method (Olsen, Cole & Watanabe 1954).

Results of the pH, EC and PO_4-P were then plotted using geographical information system (GIS) software (ArcGIS 10.2.2, Esri®) to create a visualisation of the distribution of each variable across the whole site. Data was inputted, krigged (ordinary), and mapped for spatial variability, using a circular model approach. Co-variance function measured the strength of the statistical correlation as a function of distance between each sample point; a covariance curve was then fitted to the empirical data. The model output data was used to construct a variability

map of plant-available P distribution (Fig. 6.2), see appendix 9.7.1 and 9.7.2 for EC and pH, respectively. The P distribution map clearly indicated an area (*a*) of uneven P distribution, in which the P index varied from index zero (0-9 mg kg^{-1}) to index three (26 – 45 mg kg^{-1}).

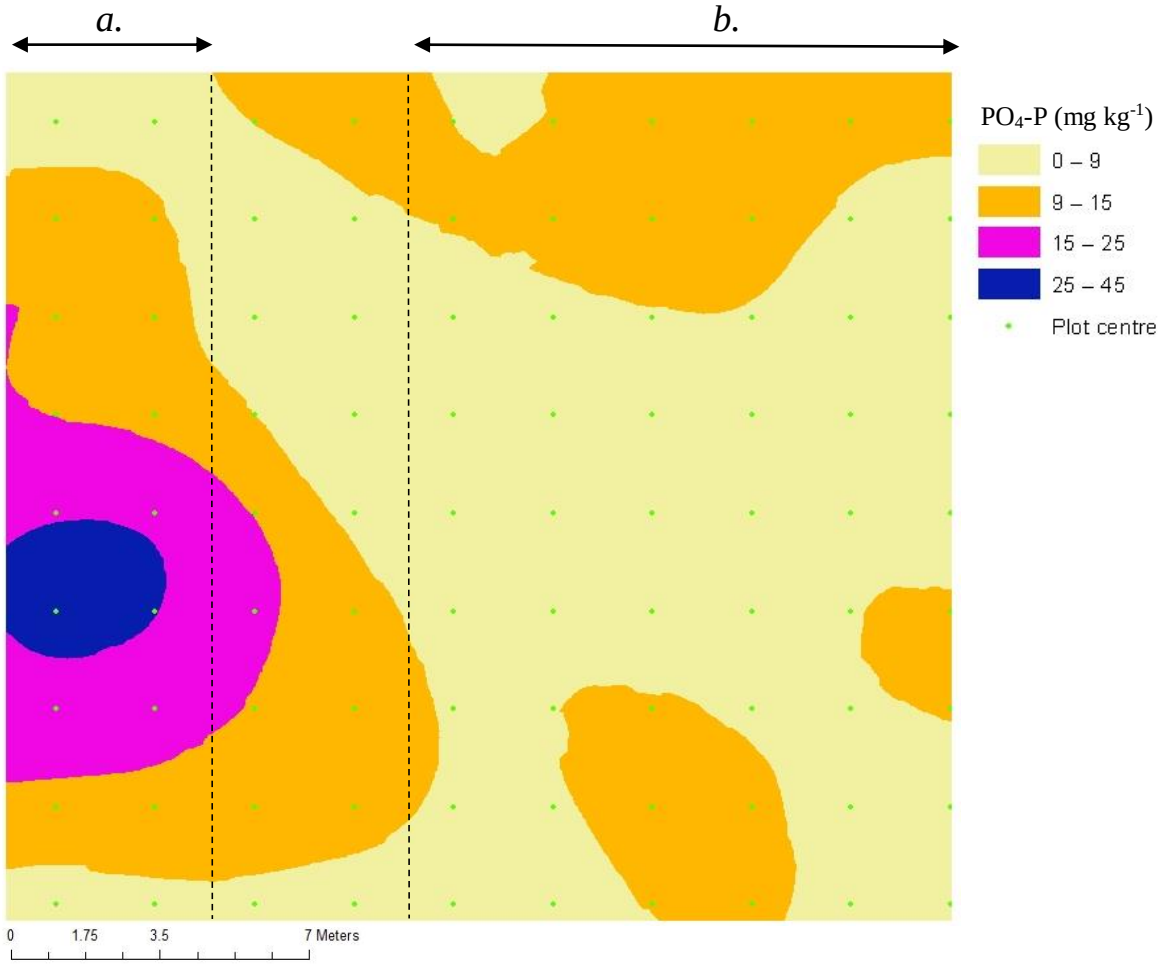

Figure 6.2. Phosphate distribution across whole field site, phosphate measured in mg kg^{-1}. Site split into two experimental sections. *a*) MG*a* P-index trial and *b)* MG*b* P-fertiliser trial

Using Fig. 6.2, the site was split into two sections, and two field trials were established:

a) P-index – examining the interactive effects between an increasing P-index and BI effectiveness and the potential to increase grass yields

b) P-fertiliser – examining the potential interactive effects between applied BIs and inorganic P fertilisers

6.4.2. Treatments

Table 6.2 gives a brief description of treatments utilised within both field trials. Elemental analysis revealed that BI application would result in only a very low input of nutrients (see Chapter 3, Section 3.5.1.1), therefore no sterilised controls were necessary.

Table 6.2. Composition and recommended application rates of bio-inoculants (BI)

BI	**Composition**		**Rec. app. rate** (kg ha^{-1})
Biagro® Grass[z] (BG)	Granule	Consortium of five MF within a vermiculite carrier < 0.5% additives	1
Single Species Inoculum[y] (SSI)	Granule	*Glomus intraradices* (BEG 72) within an attapulgite : zeolite (50:50) carrier media	1
Biagro® PhosN[z] (PN)	Liquid	Bacterial suspension containing phosphate mobilising bacteria and N fixing bacteria	250 ml

z. Commercial products supplied by Glenside Group (Livingston, UK)
y. Bespoke bio-inoculum manufactured by PlantWorks Ltd. (Sittingbourne, UK)

6.4.3. Field trials

6.4.3.1. P-index (MGa)

The mean plant-available P of the experimental site (MG*a*) was 15 mg kg^{-1} (P index 1-2). The section had a gradient of plant-available soil P, ranging from an index 0 to index 3 (Fig. 6.2). Soil samples were taken at three locations across the gradient (Fig. 6.3, marked with an asterisk) to measure total P (NRM Ltd., UK). Results of 770, 889 and 1018 mg total P kg^{-1} soil, suggested that the P gradient within the site was likely due to accidental over-fertilisation, as opposed to pedalogical differences.

The section was split into two treatment strips (BI and control); each containing nine plots, covering the range of soil P indices (Fig. 6.3). The section was seeded in September 2013, the BI treatment (Biagro® Grass, (BG)) was mixed with seed, *Lolium perenne* (Broadsward var.), at the recommended application rate (Table 6.2) and applied with a seed drill at a rate of 34 kg ha^{-1} (Oliver Seeds, UK). No fertilisers were applied at seeding. Two yield cuts were taken, in May and August 2014. Inorganic fertilisers were applied in March 2014, 150 kg ha^{-1} of N (34.4% N, ammonium nitrate) and 80 kg ha^{-1} of K (40% K, sulphated potash) (Defra. 2010). Fertilisers were applied by hand, N fertiliser was applied in two applications of 40 kg ha^{-1} followed by 110 kg ha^{-1} one week later, to prevent potential burning of fungi (Veverka, Stolcova & Ruzek 2007).

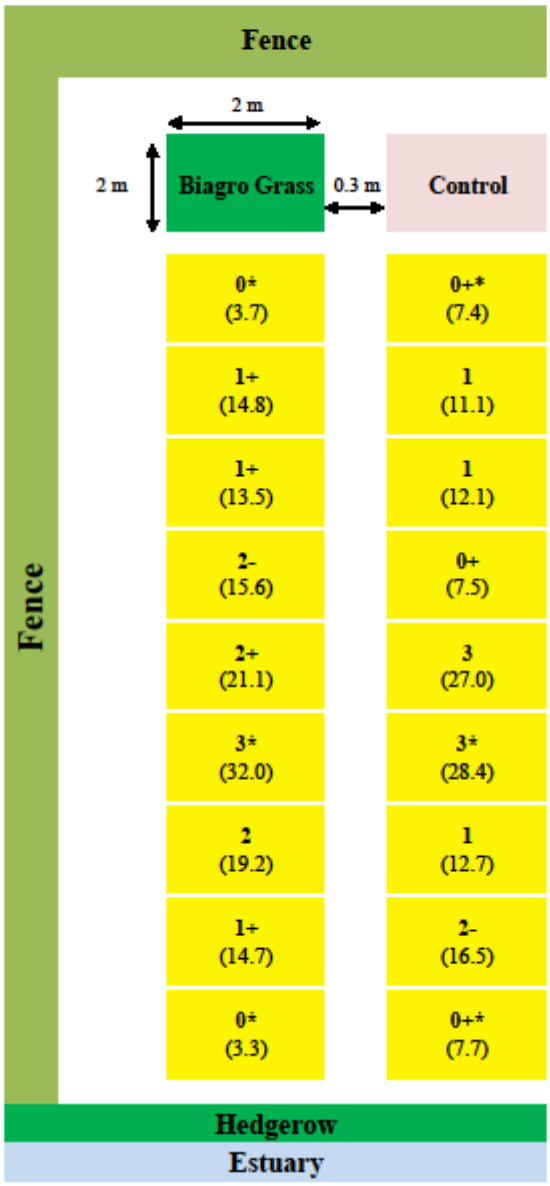

Figure 6.3: Plot layout of Morfa Ganol field trial to investigate effect of P index (*MGa*). Plots were 2 m^2 and separated by a 0.3 m buffer strip. Sampling was taken from a central 1 m^2 area within each plot. Numbers in each plot represents a soil phosphate index value (RB209). Values in parenthesis are the measured soil phosphate concentrations (mg kg^{-1}) (Olsen, 1954). Soil samples taken from plots marked with asterisk to establish total P content

6.4.3.2. P fertiliser field trial (MGb)

The mean plant-available P of the experimental site (MG*b* (Fig. 6.2)) was 8 mg kg^{-1} (P index 0). The section was seeded in July 2013, BI treatments were mixed with seed, *Lolium perenne* (Broadsward var.), and applied with a seed drill at a rate of 34 kg ha^{-1} (Oliver Seeds, UK). BIs were applied at ten times application rates (n = 9) (Table 6.2), with each BI treatment row separated by a control strip. A previous field trial, Chapter 5, indicated increased MF colonisation at ten times application rate. The site was laid out in a randomised strip design (Fig. 6.4), with each P treatment replicated within each BI treatment and control (n = 3). No fertilisers were applied at seeding.

A total of three yield cuts were taken, first yield cut taken in September 2013, 12 weeks after seeding. Inorganic fertilisers were applied in March 2014, 150 kg ha^{-1} of N (34.4% N, ammonium nitrate) and 80 kg ha^{-1} of K (40% K, sulphated potash) (Defra. 2010). Fertilisers were applied by hand, N fertiliser was applied in two applications of 40 kg ha^{-1} followed by 110 kg ha^{-1} one week later. P treatments consisted of Highland slag rock phosphate (RP, 15% PO_4), and triple super phosphate (TSP, 46% PO_4), applied at a rate of 44 kg ha^{-1} P (equivalent to 100 kg ha^{-1} P_2O_5 (Defra. 2010)), control plots had no P applied. Two further yield cuts were taken, in May and August 2014.

Figure 6.4. Plot layout of Morfa Ganol field trial (*MG6*) to investigate the effects of phosphate (P) source, triple super phosphate (TSP) and rock phosphate (RP) with bio-inoculants (BI), BG (Biagro® Grass), SSI (Single species inoculum) and PN (Biagro® PhosN). Plots were 2 m^2 and separated by a 0.3 m buffer strip. Sampling was taken from a central 1 m^2 area within each plot. P source (0 P, TSP, RP) and BI (0 BI, BG, SSI, PN), 12 treatments replicated 3 times, a total of 36 plots. All plots received nitrogen and potassium fertilisers. Grey plots had no fertiliser or treatment applications

6.4.4. Biomass measurements

For both field trials, samples of cut grass were weighed and dried in an oven (80 °C) for 24 hours to calculate dry matter yield (DMY). The DM was then dry-ashed (550 °C / 16 h) and the residue dissolved in 0.5 M HCl. The P content of the forage was then determined using the ascorbate / molybdate blue method (Murphy, Riley 1962). Total N in the forage was determined using a CHN2000 analyser (Leco Corp., St Joseph, MI). Plant samples ($n = 3$) were randomly taken from the central treatment area (1 m^2) and roots were examined for fungal colonisation; briefly, roots were rinsed, cleared using KCl and stained with Trypan blue (Phillips, Hayman 1970) and colonisation quantified using Mycocalc (Trouvelot, Kough & Gianinazzi-Pearson 1986) (see Chapter 3, Section 3.4.6-7 for full description). Final yield cut forage subsamples were also sent to Sciantec (UK) for full plant macro- / micro-elemental analysis (Al, Ca, Co, Cu, Fe, I, K, Mg, Mn, Mo, Na, S and Zn).

6.4.5. Statistical analysis

All data was analysed for statistical significance with SPSS 22.0 (IBM). Percentage data was log transformed. Data was analysed for normality using Shapiro-Wilk test. Univariate analysis indicated potential interactive effects between BI application and P-fertiliser treatment (MG*b*); Anova with post hoc tests (Fisher's LSD) analysis indicated any significant effects within each treatment (BI and P-treatment), results were considered to be significant at the $p < 0.05$ level. All data was suitably transformed, if required, to conform with Levene's test of homogeneity. Pearson's r was used to measure significant correlations within MG*a* data, the statistic was based on 1000 stratified bootstrap samples with bias correction.

6.5. Results

6.5.1. P-index trial (MGa)

There was no significant effect of BG on total grass yield (T-test, $p < 0.05$) (Table 6.3). However, analysis of individual cuts (Appendix 9.7.3), found that the second cut BG-treated grass yielded less than the control, 960 kg ha^{-1} (± 180) and 1134 kg ha^{-1} (± 182) respectively, which was almost significant ($p = 0.059$). Mycorrhizal colonisation (F%) analysis revealed all samples to be heavily colonised, with the control at 91% (± 7.8 S.E, $n = 5$) and BG at 89% (± 6.8 S.E, $n = 3$). As it was not possible within this study to differentiate between native and applied fungi, it was decided to limit fungal colonisation analysis to the first cut only.

The effect of each P index is shown in Table 6.3, though due to inadequate replicates for some of the P indices, statistical analysis performed on mean totals of all nine plots only. Yield, % P, total forage P and PER found BG-treated grass was not significantly different from the control plots (Table 6.3), however, there were some notable trends, such as the increasing % P and total forage P of the BG-treated grass with increasing P index, but decreasing PER (Table 6.3).

Table 6.3. Dry matter yield (DMY) (kg ha^{-1}), % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P) of two cuts at MG*a* per soil P index. Values in parenthesis are ± 1 standard deviation

Treatment	P index	*n*	DMY (kg ha^{-1})	% P	Total forage P (kg)	PER (g DM mg^{-1} P)
BG	0	2	6855 (± 3000)	0.19 (± 0.04)	11.6 (± 4.6)	0.58 (± 0.03)
	1	3	7470 (± 932)	0.21 (± 0.02)	13.7 (± 1.8)	0.54 (± 0.01)
	2	3	7186 (± 544)	0.23 (± 0.04)	15.3 (± 0.2)	0.47 (± 0.03)
	3	1	7722	0.25	20.0	0.39
Control	0	2	7517 (± 217)	0.16 (± 0.02)	13.1 (± 0.6)	0.58 (± 0.01)
	1	3	7003 (± 1062)	0.22 (± 0.01)	14.6 (± 3.5)	0.49 (± 0.04)
	2	3	7637 (± 1116)	0.21 (± 0.02)	14.5 (± 2.2)	0.53 (± 0.02)
	3	1	7406	0.18	15.2	0.49
Mean total						
BG		9	7267 (± 1226)	0.22 (± 0.03)	14.5 (± 3.1)	0.51 (± 0.07)
Control		9	7378 (± 826)	0.20 (± 0.03)	14.3 (± 2.2)	0.52 (± 0.04)

Correlation analysis of available soil P and several biomass measurements revealed several significant correlations for BG-treated grass (Table 6.4). Both total % P and total forage P were positively correlated with plant-available P, however this did not result in any significant yield gains (Table 6.3), and actually resulted in a negative correlation for PER. Interestingly, there were notable differences of BG-treated grass between cuts. For example the second cut DMY was significantly correlated with increased soil P, which was not in the first cut (Table 6.4).

Table 6.4. Pearson's correlation coefficients of plant-available soil phosphate (mg kg^{-1}) with DMY, % P, total forage P and PER. Statistic based on 1000 bootstrapped stratified (P index) samples (n = 9) with bias correction

Cut	Treatment	DMY	% P	Total forage P	PER
1	BG	0.101	0.881**	0.760*	-0.878**
	Control	0.213	0.362	0.357	-0.371
2	BG	0.716*	0.636	0.793*	-0.643
	Control	0.378	0.031	0.264	-0.088
Total[z]	BG	0.211	0.886**	0.791*	-0.892**
	Control	0.286	0.326	0.392	-0.341

[z] Total of cuts 1 and 2
**. Correlation is significant at the 0.01 level (2-tailed)
*. Correlation is significant at the 0.05 level (2-tailed)

6.5.2. P-fertiliser trial (MGb)

6.5.2.1. Pre-P application

The first cut after seeding, before the application of P treatments and fertilisers, showed higher yields for BI-treated grass compared to the control, but were not significant (Table 6.5). Similarly, there were no significant differences observed in any of the measured P parameters. Mycorrhizal colonisation (F%) analysis revealed all samples to be heavily colonised, the control at 91% (± 5.6 S.E, n = 9), BG at 89% (± 4.0 S.E, n = 9), SSI at 93% (± 1.0 S.E, n = 9) and PN at 91% (± 3.3 S.E, n = 9). As it was not possible, within this study, to differentiate between native and applied fungi it was decided to limit fungal colonisation analysis to the first cut only.

Table 6.5. First cut MG*b* (pre-P application) (n = 5) dry matter yield (DMY) (kg ha^{-1}), % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P). Values in parenthesis are ± 1 standard deviation

BI	**DMY (kg ha^{-1})**	**% P**	**Total forage P (kg ha^{-1})**	**PER (g DM mg^{-1} P)**
Control	2117 (± 816)	0.23 (± 0.02)	3.8 (± 0.9)	0.43 (± 0.03)
BG	2416 (± 1004)	0.22 (± 0.03)	4.8 (± 2.6)	0.47 (± 0.06)
SSI	3043 (± 1463)	0.23 (± 0.02)	5.5 (± 1.5)	0.43 (± 0.04)
PN	3511 (± 1248)	0.23 (± 0.04)	5.7 (± 2.5)	0.46 (± 0.09)

6.5.2.2. Post-P application

Individual treatment factors (BI and P source) did reveal significant differences. Two BIs significantly increased total grass yields (Table 6.6*a*); both SSI-treated grass (7056 kg ha^{-1}) and PN-treated grass (7283 kg ha^{-1}) significantly increased yields over the control (5765 kg ha^{-1}) (Anova, $p < 0.05$) with the significant effects on yield occurring in the third cut (Appendix 9.7.5). Of the P treatments, TSP application was found to significantly increase % P (0.23%) of the forage (Table 6.6b). However, the increase in % P did not lead to significant yield gains; consequently, TSP application significantly reduced PER compared to RP and no P treatments (Table 6.6*b*). There were no significant interactive effects observed between BI application and P treatments for any of the cuts.

Table 6.6*a* BI-treated grass totals (second and third cut), dry matter yield (DMY) (kg ha^{-1}) of MG*b*, % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P). Values in parenthesis are ± 1 standard deviation. Different superscript letters represent significant differences between BI treatments (n = 9) (Anova, LSD post hoc, $p < 0.05$)

BI	DMY (kg ha^{-1})	% P	Total forage P (kg ha^{-1})	PER (g DM mg^{-1} P)
Control	5765[a] (± 826)	0.21 (± 0.07)	12.2 (± 4.5)	0.51 (± 0.13)
BG	6346[a] (± 1106)	0.21 (± 0.05)	13.6 (± 5.4)	0.50 (± 0.12)
SSI	7056[b] (± 672)	0.17 (± 0.02)	11.7 (± 1.6)	0.61 (± 0.06)
PN	7283[b] (± 1537)	0.19 (± 0.05)	14.1 (± 4.8)	0.54 (± 0.11)

Table 6.6*b* P fertiliser totals (second and third cut), dry matter yield (DMY) (kg ha^{-1}) of MG*b*, % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P). Values in parenthesis are ± 1 standard deviation. Different superscript letters represent significant differences between P-source treatments (n = 12) (Anova, LSD post hoc, $p < 0.05$)

P source	DMY (kg ha^{-1})	% P	Total forage P (kg ha^{-1})	PER (g DM mg^{-1} P)
No P	6483 (± 1160)	018[a] (± 0.04)	12.7 (± 5.2)	0.57[a] (± 0.09)
TSP	6298 (± 1220)	0.23[b] (± 0.07)	14.4 (± 4.5)	0.46[b] (± 0.10)
RP	6668 (± 1118)	0.18[a] (± 0.03)	11.6 (± 2.5)	0.58[a] (± 0.09)

6.5.2.3. Nitrogen

Neither TSP nor RP significantly affected the total forage N (data not shown). Across all three cuts of grass, BI treatments did increase total forage N, compared to the control, but were not significant. However, PN-treated grass was significantly higher than the control in the 3rd cut (Table 6.7).

Table 6.7. Total forage N (kg) of forage for the three cuts (n = 3). Values in parenthesis are ± 1 standard deviation. Different superscript letters represent a significant difference (Anova, LSD post hoc, $p < 0.05$)

	Total forage N (kg ha^{-1})			
BI	**1st cut**	**2nd cut**	**3rd cut**	**Total**
Control	46.3 (± 9.4)	97.2 (± 9.8)	15.1^{a} (± 3.8)	158.6 (± 19.9)
BG	56.0 (± 13.6)	124.0 (± 24.3)	13.5^{a} (± 1.1)	193.5 (± 35.1)
SSI	80.1 (± 31.9)	104.5 (± 9.3)	18.4^{ab} (± 3.2)	202.9 (± 36.1)
PN	68.8 (± 22.9)	110.9 (± 19.9)	20.1^{b} (± 1.1)	199.8 (± 42.8)

6.5.2.4. Elemental analysis

The elemental analysis of the final cut biomass showed there to be a significant effect of BI treatment on the accumulation of three elements, Mg, Cu and I (Table 6.8). SSI significantly reduced Mg; Cu was significantly reduced by both BG and SSI treatments. The only element to see a significant increase was iodine (SSI treatment), which was also significantly reduced in BG and PN-treated grass.

Table 6.8 Magnesium, copper, iodine and chlorine content of forage (mg kg^{-1}). Values in parenthesis are ± 1 standard deviation. Different superscript letters represent significant differences between BI treatments (n = 9) (Anova, LSD post hoc $p < 0.05$)

	Elemental content (mg kg^{-1})		
BI	**Mg**	**Cu**	**I**
Control	1919^{a} (± 466)	3.63^{a} (± 0.36)	1.41^{a} (± 0.18)
BG	1756^{a} (± 298)	3.16^{b} (± 0.43)	1.05^{b} (± 0.16)
SSI	1357^{b} (± 211)	3.00^{b} (± 0.47)	1.68^{c} (± 0.22)
PN	1743^{a} (± 166)	3.36^{ab} (± 0.33)	1.16^{b} (± 0.19)

6.6. Discussion

6.6.1. P-index (MGa)

Roots of both treated and untreated grasses were found to be heavily colonised with MF, reflecting the low P status of the site (mean total of 15 mg l^{-1} PO_4-P (index 1+)), in which low soil P has been shown to be favourable for MF colonisation (Schubert, Hayman 1986, Koide 1991, Grant et al. 2005). There is a wealth of evidence to show that MF can improve productivity in soils of low fertility (Smith, Smith 2012, Smith et al. 2011, Richardson et al. 2011), and are particularly important for increasing the uptake of slowly diffusing ions such as PO_4^{3-}, and immobile nutrients such as Zn and Cu (Clark, Zeto & Zobel 1999). The P-index field trial however, did not show any significant increases in grass yield with the application of BG. Of the two cuts taken, the first cut did yield slightly higher than the control, but not significantly so. Interestingly however is the almost significant (p = 0.059) reduction in yield of the second cut of BG-treated grass. Reflecting a possible symbiotic cost of maintaining the MF, which can be as high as 20% of plant photosynthate C (Smith, Read 2008). Some studies have shown native MF to increase plant yields above that of introduced species (Maherali, Klironomos 2007, Khan et al. 2009, Williams, Ridgway & Norton 2012), which may not be acclimatised to specific soil conditions (Khan et al. 2009). While there were no significant differences in MF colonisation frequency between the treatments, it is possible that MF within BG were more energy expensive fungi for the grass to maintain than native fungi. There was also no reduction in the frequency of root colonisation with increasing P index which is somewhat contrary to the widely held view, which sees reduced MF colonisation with increasing plant-available P (Roesti et al. 2006). Recent studies have shown however how plants can maintain MF symbioses when other nutrients are limited, nitrogen for example (Nouri et al. 2014).

BG-treated grass did show trends for some of the measured P parameters, % P and total forage P, for example, were both positively correlated with increasing soil P. Suggesting the possibility of fungi, present within BG, mediating and capturing more P as availability increased. Mycorrhizal hyphae extend beyond the root rhizosphere, and effectively increase the soil zone from which P can be acquired (Clark, Zeto & Zobel 1999). However the increased acquisition of soil P by BG-treated grass did not result in any significant yield gains, the plants were ineffective in turning acquired P into dry matter; resulting in a significant negative correlation of PER with increasing soil-P. There is a possibility that introduced MF, such as

Rhizophagus irregularis found within BG, were not as effective as the native strains. Significant plant / MF co-adaptation to local nutrient conditions has been shown (Johnson et al. 2010, Antunes et al. 2012); and the negative correlation with PER, as seen on BG-treated grass plots, is in line with other studies in which MF plant yield enhancement has been shown to be compromised with increasing plant-available P (Schubert, Hayman 1986, Koide 1991, Grant et al. 2005). The increased P acquisition and lack of DMY would suggest that yield limitation was not driven by P. Control grass roots, were found to be similar for all measured parameters as BG-treated grass. Indeed by the end of the trial, although not significant, total yield for control plots was higher than the BG-treated plots. Soil P appeared to play no significant role with regards to the DMY, % P of the biomass, total forage P and PER of the control plots, which all exhibited non-significant correlations.

The second cut, however, BG-treated grass was found to have a positive correlation between plant-available P and both DMY and total forage P. Overall, this suggests that while P was mediating a positive growth response, yield was limited due to another factor, N for example. No inorganic fertilisers were applied prior to the second cut.

6.6.2. P-source (MGb)

Results of the second trial, examining the interaction of inorganic P fertilisers and BIs, did show an effect of inorganic P fertiliser application on some grass growth parameters. For example, TSP, unsurprisingly, significantly increased the % P content of the grass more than RP and zero P treatments, however this did not result in any significant increases in DMY. Consequently there was, with TSP application, a significant reduction in PER. The increase in the % P and non-concurrent increase in DMY would suggest luxury uptake of P by the grass, and growth limitation was driven by another mechanism other than P limitation.

There were no interactive effects between BI and P-source, unlike the laboratory where interactive effects were seen (Chapter 3). Highlighting both variability and subsequent difficulty in testing of BIs between the laboratory and field. For example, whilst the field site was very low in plant-available nutrients such as N and P, applied fertilisers may not have been as effective due to weather conditions (very wet shortly after application) and the sandy nature of the soil, wich possibly combined to see much of the applied fertilisers, especially N, wash away and fail to be taken up by the grass. That said there were significant BI treatment effects, with both SSI- and PN-treated grasses yielding higher than the control and BG-treated grass. However, with no significant increases in total forage P, the results would suggest that yield

increases were not a P-effect and point to the potential of SSI and PN to alleviate nutrient limitation, other than P. MF have been shown to increase plant competiveness for N resources for example (Ruzicka et al. 2012), whilst PN contains N fixing micro-organisms.

Within this study P was initially considered to be the only limiting nutrient, as such MF / plant symbiosis was expected to be one of mutualism, the bi-directional exchange of C and P. The law of minimum is an established rule regards plant growth limitation, in which plant growth is controlled by the most limited resource (von Liebig 1842). However studies have shown that this is not always the case as plants can be co-limited by multiple resources (Johnson et al. 2015); and any symbiosis between MF and plant can change with respect to which resource is limiting. Johnson (2015) was able to demonstrate that in P-limited systems the traditional mutualistic plant-fungi continuum prevails, whereas in an N-limited system the relationship veers more towards one of commensalism or even parasitism. The N : P ratio of plant biomass can give an indication as to which nutrient is limiting, a ratio < 14 is considered to be indicative of N limitation, whilst > 16 a sign of P limitation (Koerselman, Meuleman 1996).

After N fertilisation and P treatment application, the N : P of the plant biomass changed potentially altering the nature of the MF / plant symbioses. N : P values for all treatments were below 14, indicating that plants were potentially N limited. TSP application saw a significant reduction in the second cut with an N : P of ~ 9, compared to 13 for both RP and zero P treatments (Appendix 9.7.7), TSP application exacerbating N limitation through potentially reducing the value of the MF symbioses. The sudden increase in soluble P, from TSP application, was likely transferred to the plant biomass as per the mutualism expectation for plant / fungi in P limited soil, however the lack of N uptake and subsequent plant growth led to an increase in % P concentration, and thus reduced the N : P ratio. Even though inorganic N fertilisers were applied, they failed to alleviate the N limitation sufficiently, the sandy loam soil was very dry on application, and shortly after there was a large amount of rainfall which may have removed large quantities of the applied N. Also MF fungi have been shown to satisfy their own N requirements before releasing surplus N to a host plant (Hodge, Storer 2015). Concentrations of N within MF hyphal tissue can be ten times that of plant roots (Hodge & Fitter, 2010), as such there can exist competition between MF, other soil micro-organisms and plants for N resources. That said total N uptake, measured as the total N content of the forage, found totals were higher for BI-treated grass than controls. The third cut was significantly higher for PN-treated grass. PN contains N-fixing species, such as azotobacter and azospirillum, which are able to provide N for plant growth through atmospheric N fixation.

The increased N uptake may have contributed to the significantly higher DMY observed for the third cut PN-treated grass (Appendix 9.7.6), which also suggest that the BI may have persisted within the soil or exerted a priority effect, in which initial populations of species have been shown to determine final community composition (Verbruggen et al. 2013, Mummey, Antunes & Rillig 2009).

There were also yield differences between single (SSI) and consortium (BG) BIs, the former yielding significantly higher; suggestive of potential variability in proliferation success of micro-organisms contained within each BI. The ability of applied micro-organisms to proliferate within an existing community is pivotal for the overall efficacy of any BI product. Roots of the control plants were heavily colonised and a potential indication of the proliferation of native MF species within the soil, which may compete with any applied MF, potentially undermining the efficiency of the applied BIs. The success of applied micro-organisms is subject to numerous other soil, crop and environmental factors, from crop species compatibility, size and effectiveness of indigenous microbial populations to soil fertility and management (Adholeya, Tiwari & Singh 2005). Soil pH, for example, has been shown to be a major factor in selecting for microbial community structure (Rousk et al. 2010). Within this study, pH across the whole site was found to be within a range of 1 pH unit (Appendix 9.7.2), which has been shown to have less impact on microbial community structure compared with P pool availability (DeForest et al. 2012).

Single species PMM can have beneficial effects (Jansa, Smith & Smith 2008, Roesti et al. 2006), but mixed inocula have been shown to be more flexible and productive within variable abiotic and biotic environments (Malusá, Sas-Paszt & Ciesielska 2012), as a diverse community of fungi and associated bacteria are able to supply many macro- / micro-nutrients (Hart, Forsythe 2012). For example, within this trial, the foliar content of iodine was significantly reduced with BG compared to SSI application. The variations observed were likely due to the availability of iodine within the soil. Availability of iodine is greatly influenced by reactions with soil components such as sorption (Whitehead 1975). Micro-organisms can alter root rhizosphere processes, such as pH, which affects sorption kinetics and uptake of various elements (Illmer, Schinner 1992), functional diversity within MF with respect to the uptake of various elements has been shown (Veresoglou, Shaw & Sen 2011).

Some studies have shown similar decreases in foliar nutrients, citing the mycorrhizal dependency of the grass and availability of P as a potential cause (Hart, Forsythe 2012). *Lolium*

perenne, a C3 grass, has a reduced mycorrhizal dependency than C4 plants, such as *Zea mays* (Wilson, Hartnett 1997). The photosynthetic pathway utilised is less P-demanding than within C4 plants (Bueckert 2013); removing nutrient limitation, through inorganic fertiliser application, may have reduced MF effectiveness on the uptake of other macro- / micro-nutrients.

6.7. Conclusion

This study highlighted the difficulties in field testing BI efficacy, in which results were found to be highly variable. While the study was able to show that the application of some BIs , single species MF inoculant and Biagro® PhosN, increased dry matter yields of *Lolium perenne* on P limited soils. With no significant interaction between BIs and phosphate fertiliser, yield increases were more likely due to increased uptake of nitrogen and increased water uptake. The bacterial BI, Biagro® PhosN, which increased N uptake significanlty, could potentially form part of a dual application of MF and bacterial suspension.

The study did highlight variations between single and multiple species BI applications, in which the single species MF inoculant yielded higher than the consortia of MF species (Biagro® Grass). This could ahave implications in future BI design. The study did highlight however the potential of Biagro® Grass to aquire P from legacy P, but that increased available P was shown to reduce the effectiveness of the BI on grass yields. Field conditions may not have been optimum for a true reflection of the BIs effectiveness, as such further field testing is required. Micro nutrient analysis alluded to potential variations between BI applications. A closer examination of below ground microbial interactions will better inform as to the variations between native and applied single and consortium BIs that the above ground biomass alluded to.

BIs can form part of a nutrient management program, but is dependent on N and P status of the soil prior to BI application. Further work is required to improve effectiveness and consistency of BIs across variable abiotic and biotic soil environments.

6.8. References

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CHAPTER 7: ARTICLE V

Examining changes in soil fungal communities of a ryegrass mix under varying management practices using next generation DNA sequencing

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7.1. Abstract

Fungi are integral component of the soil ecosystem, providing many services from pathogen suppression to nutrient cycling enhancement. The plant benefits afforded by fungi have seen them exploited commercially as bio-inoculants (BIs). The efficacy of any applied fungi are however subject to numerous soil, crop and environmental factors, such as the size and effectiveness of indigenous microbial populations, soil fertility to the application of inorganic fertilisers.

Advances in DNA-based techniques have allowed researchers to explore the dynamic role of fungi within the soil in ever greater detail. In recent years there has been an increase in the use of next generation DNA sequencing (NGS). In this work, we have undertaken a study of the potential changes in soil fungal assemblages under varying management practices. The effects of inorganic fertiliser applications (ammonium nitrate, triple super phosphate and rock phosphate), sampling date (July and September) and bio-inoculant application were all assessed using the NGS Ion Torrent™ molecular technique. Single and consortia BI formulations were applied to a *Lolium* grass mix (Broadsward var.) and three field trials (Gadlas and Morfa Ganol (*a* & *b*)) were established in 2012 and 2013.

Results indicated that the Ion Torrent™ NGS platform was able to discern clear differences in fungal abundances of soils under varying treatments. Fungal assemblages were found to be significantly affected by both treatment (BI application and inorganic nitrogen) soil type and sampling date, for example *Mortierella* sp. (phylum Fungi incerate sedis; order Mortierellales) was found to be significantly reduced with the application of nitrogen fertiliser. Phylum abundances were found to be significantly affected by sampling date at one of the sites, Morfa Ganol, which exhibited a reduction in Basidiomycota abundance in September compared with July; while at the Gadlas site, an increase in Ascomycota abundance was observed after the application of ammonium nitrate, which subsequently significantly affected the overall fungal diversity and equitability of the soil. Nitrogen fertiliser was the only treatment to significantly affect fungal diversity and equitability measures.

The study was able to show the potential of NGS technology, Ion Torrent™, for examining changes in fungal communities within the field.

7.2. Introduction

Fungi are an integral part of the soil ecosystem providing services such as the decomposition of organic wastes and crop residues (Javaid 2009), pathogen suppression (Azcon-Aguilar, Barea 1996), nutrient cycling enhancement via mycorrhizas (Barea, Azcon & Azcon-Aguilar 2002), improved soil aggregation (Rillig, Mummey 2006), detoxifying pesticides and the production of bioactive compounds (Singh, Pandey & Singh 2011); all of which contribute to improving soil quality. The plant benefits afforded by fungi have seen them exploited commercially as bio-inoculants (BIs), and has seen the global market grow ~ 10% per annum (Berg 2009); valued at $440 million in 2012 and expected to reach $1,295 million by 2020 (Transparency Market Research 2014). Much of the increased utilisation of BI products is, in part, a drive to reduce reliance and consumption of depleting rock phosphate reserves, the raw material used in the manufacture of inorganic phosphate fertilisers (Van Kauwenbergh 2010, Cordell, White 2011).

The efficacy of any applied fungi are subject to numerous soil, crop and environmental factors: crop species compatibility, size and effectiveness of indigenous microbial populations, soil fertility and management (Adholeya, Tiwari & Singh 2005), priority effects, in which initial populations of species determine final community composition (Mummey, Antunes & Rillig 2009, Verbruggen et al. 2012), organic and inorganic fertiliser applications (Egerton-Warburton, Johnson & Allen 2007, Lazcano et al. 2013). Collectively, these affect the soil microbial dynamics, functional processes and hence performance of commercial BIs. BI design frequently utilises many genera of mycorrhizal fungi (MF), offering functional redundancy and / or added plant benefits. Single species plant growth-promoting MF can have beneficial effects (Roesti et al. 2006, Jansa, Smith & Smith 2008), but mixed inocula have been shown to be more flexible and productive within variable abiotic and biotic environments (Malusá, Sas-Paszt & Ciesielska 2012).

Inoculation consists of supplying high densities of viable and efficient microbes for a rapid colonization of the host rhizosphere, in which fungal populations of the host root rhizosphere are significantly altered compared to the bulk soil (Marilley et al. 1998), as such BI inoculation could induce at least a transient perturbation of the equilibrium of soil microbial communities (Trabelsi, Mhamdi 2013). However application rates of BIs can be as low as < 1 kg ha^{-1}, which has led to some scepticism of claims of plant benefits from commercial BIs (Owen et al. 2015).

Few studies have examined bulk soil fungal population changes (Lumini et al. 2010, Borriello et al. 2012, Davison et al. 2012), with many studies of MF communities within field soils, using both molecular and morphological techniques, focusing on mycorrhizal roots (Alguacil et al. 2010, Verbruggen et al. 2010) or spores (Oehl et al. 2004, Egerton-Warburton, Johnson & Allen 2007).

Advances in DNA-based techniques have allowed researchers to explore the dynamic role of fungi within the soil in ever greater detail. In recent years there has been an increase in the use of next generation DNA sequencing (NGS), in part due to a reduction in the costs of the technology, which were prohibitive for larger sample sets (Egerton-Warburton, Johnson & Allen 2007). High-throughput sequencing methods enable detailed, quantitative analysis of fungal communities in large sample sets and provide ecological information that extends far beyond that provided by previous methods in terms of detail and magnitude. Ion Torrent™ is an NGS platform which uses semiconductor sequencing technology. It is a method of sequencing by synthesis during which a complementary strand is built based on the sequence of a template strand. Each time a nucleotide is incorporated into the DNA a proton is released, and the subsequent change in pH is measured by pH-sensitive field effect transistor. Therefore, no labelled nucleotides are used and synthesis is detected directly. It offers shorter run times when compared to systems based on fluorescence detection for example. One of the limitations of the technology is the short read length, of ~ 200 base pairs, although there are now 400 base pair chips available and, in line with Moore's law, increased chip density in the future will lead to increased read lengths. With a read length of 200 base pairs, the Ion Torrent™ within this study utilises a novel approach focusing on the D1 region of the large subunit (LSU) 26S rRNA gene, which has been shown to provide good resolution to the genus level (Cole et al. 2014).

In this work, we have undertaken a study of the potential changes in soil fungal assemblages under varying management practices. The effects of inorganic fertiliser applications, sampling date and bio-inoculant application were all assessed using the NGS Ion Torrent™ molecular technique. Single and consortia BI formulations were applied to a *Lolium* grass mix (Broadsward var.) and three field trials were established in 2012 and 2013. Soils of the field trials were sampled across a growing season. Samples were taken before and after inorganic fertiliser applications of nitrogen and phosphate. Inorganic fertilisers such as triple super phosphate and ammonium nitrite, whilst providing essential plant nutrient, P and N

respectively, also have been shown to alter microbial soil populations within the bulk soil (Egerton-Warburton, Johnson & Allen 2007, Paungfoo-Lonhienne et al. 2015).

7.3. Aims

- To examine changes in soil fungal community assemblages abundance and diversity in two soil types at two sampling dates

- To assess the impact of inorganic fertilisers, ammonium nitrate, triple super phosphate and rock phosphate on the soil fungal community assemblages, abundance and diversity

- To examine the effect of bio-inoculants on soil fungal community assemblages, abundance and diversity

7.4. Methodology

7.4.1. Sites

Field trials were established at two sites within Henfaes Research Centre (Fig 7.1), Abergwyngregyn, UK (53°14′ N, 4°01′ W), which is characterized as having a temperate-oceanic climate regime. The soil, a Eutric Cambisol, developed since the last glacial period (10,000 ybp). The site has a mean annual temperature of 9.8 °C, and mean annual rainfall of 800 mm (Farrell et al. 2011).

Figure 7.1. Location of field trials at Henfaes Research Centre, *a*) Gadlas (GA) *b*) Morfa Ganol (MG) (Imagery ©2012, Infoterra Ltd & COWI A/S, DigitalGlobe, GeoEye, Getmapping plc, Map data ©2012 Google)

Sites were re-seeded in August 2012 (GA), MG was split into two field trials (section 7.3.1.2), July 2013 (Site MG*b*) and September 2013 (site MG*a*), at 34 kg ha^{-1} (Oliver Seeds, UK) using a seed spreader. Grass seed used was the variety Broadsward (Oliver Seeds, UK); a mix of perennial and hybrid ryegrasses (*Lolium* spp.). BI treatments were mixed with seed. Table 7.1 gives a brief description of BI treatments utilised within both field trials. Elemental analysis revealed that BI application would result in only a very low input of nutrients (see Chapter 3, Section 3.5.1.1), therefore no sterilised controls were necessary.

Table 7.1. Composition and recommended application rates of bio-inoculants (BI) used in all field trials

BI	**Composition**		**Rec. app. rate (kg ha^{-1})**
Biagro® Grass[z] (BG)	Granule	Consortium of five MF (from the phylym Glomermycota[x]) within a vermiculite carrier < 0.5% additives	1
Biagro® MP[z] (BMP)	Powder	Consortium of MF and phosphate mobilising bacteria	0.150
Biagro® S[z] (BS)	Powder	Consortium of MF and bacteria with humates, algal extracts and amino acids	0.125
Single Species Inoculum[y] (SSI)	Granule	*Glomus intraradices* (BEG 72) within an attapulgite : zeolite (50:50) carrier media	1
Biagro® PhosN[z] (PN)	Liquid	Bacterial suspension containing phosphate mobilising bacteria and N fixing bacteria	250 ml

z. Commercial products supplied by Glenside Group (Livingston, UK)
y. Bespoke bio-inoculum manufactured by PlantWorks Ltd. (Sittingbourne, UK)
x. personal comm. PlantWorks Ltd. (Sittingbourne, UK)

7.4.1.1. Gadlas (GA)

A long term ley (25 years), originally seeded with *Lolium perenne* and *Trifolium repens* (60 : 40); with an annual split dose application of 120 kg ha^{-1} N and 20 kg ha^{-1} P. When ploughed, for the trial, the original sown cultivars had worn out naturally and ingress of secondary grasses such as *Agrostis stolonifera*, *Holcus lanatus* and *Poa annua* dominated the sward. Soil characterised as a brown earth loam, with a mean plant available P content of 21mg kg^{-1}, the main soil chemical characteristics were analysed by Glenside (Livingston, UK) and NRM (Berkshire, UK), and are presented in Table 7.2.

Table 7.2. Soil characteristics, pH, cation exchange capacity (CEC), organic matter (OM), elemental content (mg kg^{-1}) (Glenside Albrecht ®, Livingston, UK. ISO/IEC 17025-2005). Nitrate (NO_3) and ammonium (NH_4), with total and plant-available phosphorus (P) and total potassium (K) (NRM Ltd., Berkshire, UK). Values are of the entire field site

			N*		P		K
pH	**CEC (meq 100 g^{-1})**	**OM (% w/w, LOI**)**	**NO_3-N (mg kg^{-1})**	**NH_4-N (mg kg^{-1})**	**Total (mg kg^{-1})**	**PO_4-P^x (mg kg^{-1})**	**Total^y (mg kg^{-1})**
5.5	14.65	5.6	12	23	1199	21	862

Elemental content (mg kg^{-1})

Ca	Mg^z	Na	Fe	Mn	Cu	Zn	Cl	I	Mo
3359	254	125	827	184	6.4	18.9	52	0.09	2.24

**loss on ignition
*Total N measured at 0.28% (w/w), of which 139 kg ha^{-1} N available (30 cm profile)
[1]agricultural P index of 2
[2]agricultural K index of 2+
[3]agricultural Mg index of 2

The site was laid out in a randomised strip design (Fig. 7.2), with each BI treatment replicated within each application rate row (recommended and ten times application rates) (n = 3). Rows were separated by a two meter buffer strip. No fertilisers were applied at seeding (August 2012). Eight weeks after seeding an establishment cut was taken (October 2012). 50 kg ha^{-1} of N (34.4% N, ammonium nitrate) was applied in June 2013, in two applications of 25 kg ha^{-1} applied seven days apart. Fertiliser application rates were split into two and were slightly below the RB209 recommendations of 60-70kg ha^{-1} to prevent potential detrimental effects on fungi within the soil (Veverka, Stolcova & Ruzek 2007), which would affect later analysis of fungal soil communities. First yield cut was taken in July 2013. A further 50 kg ha^{-1} of N and K (potash) was applied after the first yield cut. The second yield cut was taken in September 2013.

Soil samples consisted of bulk soil, whilst some roots and subsequent rhizosheric soil will have also formed part of the sample, the majority of the sample was of bulk soil. Soil samples, five cores (10 cm depth) from within a 1 m^{-2} area within each plot and combined together (~ 200 g soil); Soil samples were taken in June 2013 before and after the application of 25 kg ha^{-1} N, July 2013 after the second yield cut and from the control plots in September 2013; buffer strips were sampled in July and September 2013.

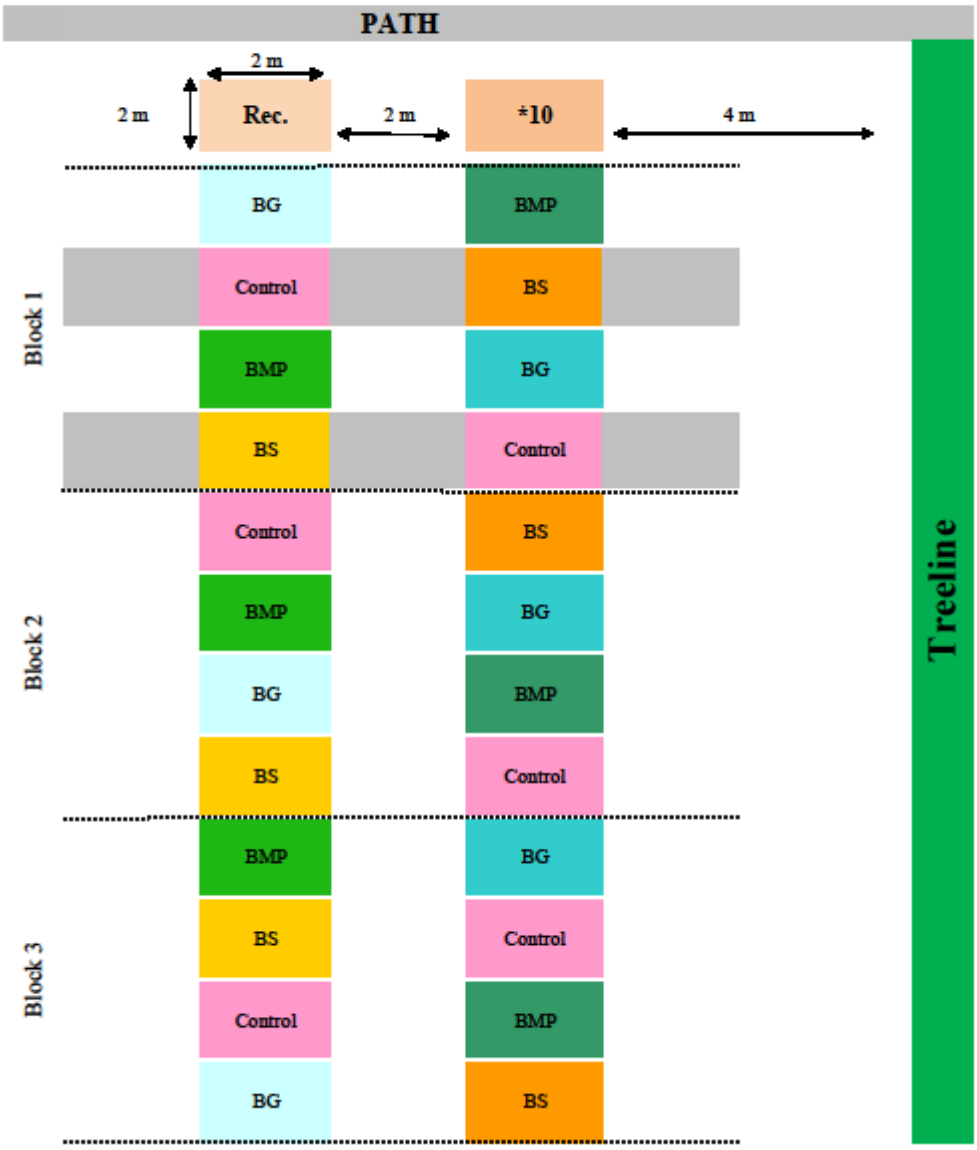


Figure 7.2: Plot layout of Gadlas field trial (GA). Plots were 2 m^2 and separated by a 2 m buffer strip. Sampling was taken from a central 1 m^2 area within each plot. Grey plots had no fertiliser or treatment applications. Each strip consisted of three blocks of three BI treatments (Biagro Grass (BG), Biagro MP (BMP), Biagro S (BS)) and a control, a total of 12 plots per application rate (recommended (Rec) and ten times recommended (*10))

7.4.1.2. Morfa Ganol (MG)

Previous site history included *Lolium perenne* with *Trifolium repens* and chicory (*Cichorium intybus*) (2010), followed by potato (*Solanum* var.; 2011) and barley (*Hordeum vulgare* L.; 2012). Soil characterised as a sandy loam, with a mean plant-available P content of 12 mg kg^{-1}, the main soil chemical characteristics were analysed by Glenside (Livingston, UK) and NRM (Berkshire, UK), and are presented in Table 7.3.

Table 7.3. Soil characteristics, pH, cation exchange capacity (CEC), organic matter (OM) and elemental content of the soil (mg kg^{-1}) (Glenside Albrecht ®, Livingston, UK. ISO/IEC 17025-2005). Nitrate (NO_3) and ammonium (NH_4), with total and plant-available phosphorus (P) and total potassium (K) (NRM Ltd., Berkshire, UK)

			N*		P		K
pH	**CEC (meq 100 g^{-1})**	**OM (% w/w, LOI**)**	**NO_3-N (mg kg^{-1})**	**NH_4-N (mg kg^{-1})**	**Total (mg kg^{-1})**	**PO_4-P^x (mg kg^{-1})**	**Total^y (mg kg^{-1})**
5.8	7.66	2.8	5.3	6.2	500	12	595

Elemental content (mg kg^{-1})

Ca	Mg^z	Na	Fe	Mn	Cu	Zn	Cl	I	Mo
2088	123	77	1093	152	4.3	9.2	28	0.15	1.06

**loss on ignition
*Total N measured at 0.26% (w/w), of which 46 kg ha^{-1} N available (30 cm profile)
^{x}agricultural P index of 1
^{y}agricultural K index of 2-
^{z}agricultural index of 2

Variations in plant P availability within the site allowed for the site to be split into two trials. MG*a* with a gradient of P availability ranging between 3.7 – 32 mg kg^{-1} and site MG*b* with a mean plant-available P of 8 mg kg^{-1}. Inorganic fertilisers were applied in March 2014, 150 kg ha^{-1} of N (34.4% N, ammonium nitrate) and 80 kg ha^{-1} of K (40% K, sulphated potash) (Defra. 2010). BI treatments were applied at ten times application rates, previous work had demonstrated a significant effect on mycorrhizal colonisation at this application rate (Chapter 5, Section 5.5.1).

7.4.1.2.1. Morfa Ganol – P-index (MGa)

The section was split into two treatment strips (Biagro® Grass (BG) and control), each containing nine plots, covering a range of soil P indices (Fig. 7.3). The section was seeded in

September 2013, the BI treatment was mixed with grass seed, *Lolium perenne* (Broadsward var.), at the recommended application rate (Table 7.1) and applied with a seed drill at a rate of 34 kg ha^{-1} (Oliver Seeds, UK). No fertilisers were applied at seeding. Two yield cuts were taken, May and August 2014. Soil samples were taken in August 2014.

7.4.1.2.2. Morfa Canol – Inorganic phosphate (MGb)

The site was laid out in a randomised strip design (Fig. 7.4), with each P treatment replicated within each BI treatment and a control (n = 3). A total of three yield cuts were taken, first yield cut taken in September 2013, 12 weeks after seeding. P treatments applied in March 2014 consisted of Highland slag rock phosphate (RP, 15% PO_4), and triple super phosphate (TSP, 46% PO_4), applied at a rate of 44 kg ha^{-1} P (equivalent to 100 kg ha^{-1} P_2O_5 (Defra. 2010)), control plots had no P applied. Soil samples consisted of bulk soil, whilst some roots and subsequent rhizosheric soil will have also formed part of the sample, the majority of the sample was of bulk soil. Soil samples, five cores (10 cm depth) from within a 1 m^2 area within each plot and combined together (~ 200 g soil); Soils were sampled in July and September 2013 (control plots), April 2014 and August 2014 (after NK application); buffer strips were sampled in August 2014.

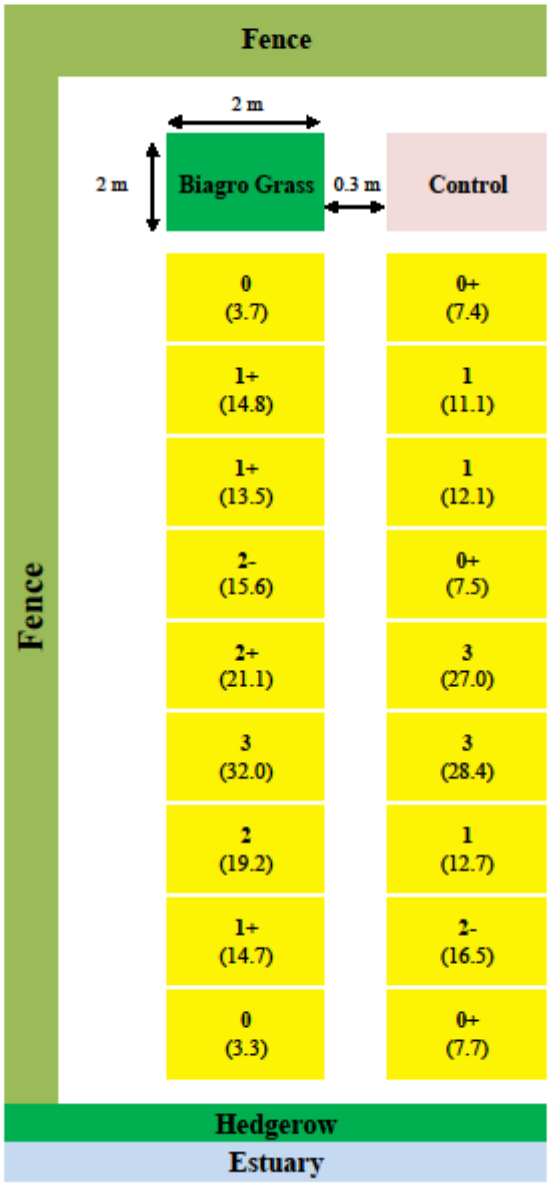


Figure 7.3: Plot layout of Morfa Ganol field trial to investigate effect of P index (*MGa*). Plots were 2 m^2 and separated by a 0.3 m buffer strip. Sampling was taken from a central 1 m^2 area within each plot. Numbers in each plot represents a soil phosphate index value (RB209). Values in parenthesis are the measured soil phosphate concentrations (mg kg^{-1}) (Olsen, 1954)

Figure 7.4. Plot layout of Morfa Ganol field trial (*MG6*) to investigate the effects of phosphate (P) source, triple super phosphate (TSP) and rock phosphate (RP) with bio-inoculants (BI), BG (Biagro® Grass), SSI (Single species inoculum) and PN (Biagro® PhosN). Plots were 2 m^2 and separated by a 0.3 m buffer strip. Sampling was taken from a central 1 m^2 area within each plot. P source (0 P, TSP, RP) and BI (0 BI, BG, SSI, PN), 12 treatments replicated 3 times, a total of 36 plots. All plots received nitrogen and potassium fertilisers. Grey plots had no fertiliser or treatment applications

7.4.2. Ion Torrent™

7.4.2.1. Soil preparation and DNA extraction

Soil samples were frozen (-80 °C) within one hour of sampling. Frozen soil samples were freeze dried for 48 hours, samples were then ground (< 1 mm). Sieved soil was thoroughly homogenised and 200 mg added to DNA extraction tubes. DNA extracted using PowerSoil® DNA extraction kit (MoBio Laboratories, Solana, CA, USA), as directed by the manufacturer.

7.4.2.2. PCR

PCR of the fungal DNA from the PowerSoil® extraction was amplified using the D1F2 forward primer (Table 7.4) and the NILC2 AF reverse primer (Table 7.5). The forward primer was linked at the 5' end to a barcode sequence and a different barcode was used for each sample to allow for multiplexing during sequencing. In addition the forward primer consisted of a calibration sequence and an Ion Torrent™ adaptor and the reverse primer was linked at the 5' end to an Ion Torrent™ adaptor.

Table 7.4. Forward primer configuration

Ion Torrent™ sequence primer	5'-CCATCTCATCCCTGCGTGTCTCCGAC
Calibration sequence	TCAG
Barcoded primer, example.	CTAAGGTAACC
D1F2 primer	YYAGTARCTGCGAGTGAAG-3'

Table 7.5. Reverse primer configuration

Ion Torrent™ primer used to anneal beads during emulsion PCR	5' – CCTCTCTATGGGCAGTCGGTGAT
Reverse primer, NILC2 R1	GAGCTGCATTCCCAAACAA – 3'

The PCR product was amplified using the cycle: denaturing at 94 °C for 5 minutes followed by 30 cycles consisting of denaturing 94 °C 30 seconds; annealing 52 °C 30 seconds; extension 72 °C for 30 seconds followed by a final extension step for 5 minutes.

The target DNA sequence for the primers is the D1 variable region (approximately 200 base pairs) of the large sub-unit (LSU) of ribosomal DNA (Fig. 7.5). D1 / D2 region enables both

genus and phylogenetic identification using the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/) classifier, and gives good resolution to genus level for the majority of fungal taxa (Cole et al. 2014).

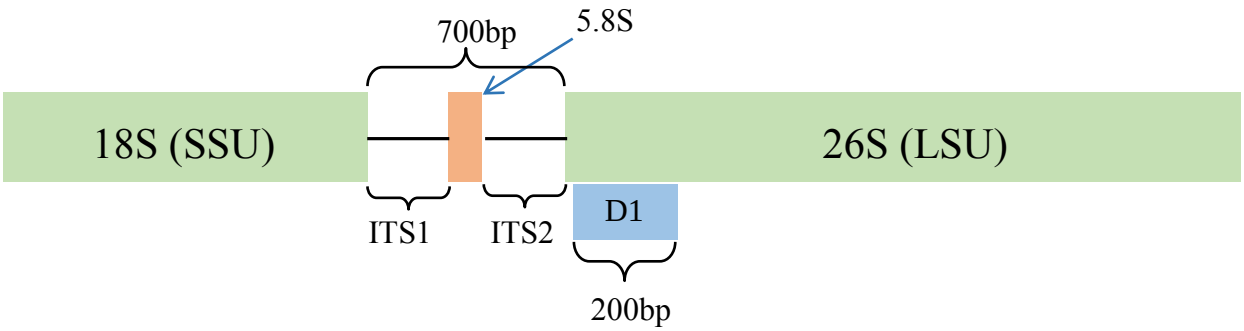


Figure 7.5. The D1 region of the Large SubUnit (LSU) (26S) targeted by DIF2 and NILC2 primers

7.4.2.3. DNA clean-up

Clean-up of the DNA was carried out using NBS Biologicals (Huntingdon UK) Spin Column PCR purification kit (NBS664), according to the manufacturer's protocol. Following this, quantification of the DNA extract was carried out using nanodrop quantification, using a Thermo Scientific NanoDrop 1000 Spectrophotometer V3.7 (Wilmington, DE, USA). Samples were pooled to equimolar concentrations. To further purify samples, an Invitrogen E-gel (Paisley, UK) was carried out to extract the DNA bands of correct length of ~300 base pairs. DNA was then assessed for purity, and to get an accurate quantification for the emulsion PCR step, using an Agilent 2100 Bioanalyzer (Santa Clara, CA) with an Agilent High Sensitivity DNA chip (Agilent reference 5067-4626) (Appendix 9.8.1). The concentration of the pooled sample was adjusted to 12 pM following a two-step dilution.

7.4.2.4. Emulsion PCR

The emulsion PCR was run using the Ion PGM™ Template OT2 solutions kit (Ion Torrent™ reference 4481105) and supplies kit (Ion Torrent™ reference 4480981) with the Ion Torrent™ One Touch 2 emulsion PCR machine according to the manufacturer's instructions. Our protocol differs in the target pM concentration used i.e. 12 pM to avoid polycolonal beads, rather than the instructed 26 pM concentration, this has been derived empirically from earlier runs.

7.4.2.5. Enrichment and Sequencing

The enrichment process was carried out on the Ion Onetouch™ ES (enrichment system) to remove any beads with no DNA strands attached and is carried out according to the manufacturer's instructions. Samples were then sequenced using an Ion PGM™ Sequencing 200 Kit v2 (Ion Torrent™ reference 44820007) in association with the Ion Torrent™ PGM which was initialised according to the manufacturer's protocol. A 316-V2 chip (Ion Torrent™ reference 4483188) was loaded according to the manufacturer's protocol.

7.4.2.6. Data Processing

The sequencing data was downloaded from the Ion Torrent™ server as BAM format. Bam is a binary version of sequence alignment map (SAM). Data was unpacked from BAM data to FASTA (standard text format) and QUAL (quality information) files, using the PICARD software package (http://picard.sourceforge.net). Data was quality checked using MOTHUR (http://www.mothur.org). A quality score average 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, and checked for chimeras using the UCHIME function of USEARCH (http://www.drive5.com/usearch). Sequences were de-replicated (discarding singletons), sorted and OTUs clustered using USEARCH / UPARSE (v7 (Edgar 2013)). A taxonomy was assigned to each OTU using the Ribosomal Database Project (RDP) naïve Bayesian classifier against a curated fungal LSU database (Wang et al. 2007), where genus could not be assigned using the classifier it was set to the OTU identifier. Data were then rendered in Excel to give relative abundances of the assigned taxa for each quadrat any non-fungal taxa were reported separately.

A spread sheet of phyla and quantities was produced for analysis. Equation 1*a* was used to calculate Shannon diversity index (*H*), equation 1*b* was used to calculate equitability index (E_H)

Equation 1*a*. $H = -\sum_{j=1}^{S} p_i \ln p_i$ *b*. $E_H = \frac{H}{\ln S}$

Where: p_i = fraction of the entire population made up of species *i* (proportion of a species *i* relative to total number of species present, not encountered) S = numbers of species encountered. Equitability (E_H) assumes a value between 0 and 1 with 1 being complete evenness.

7.4.3. Statistical analysis

All data were analysed using Past (v.3.06) (http://folk.uio.no/ohammer/past) and SPSS 22.0 (IBM). Phylum percentage data was log transformed and the data checked for normality (Shapiro-Wilk) and where appropriate analysis of variance was performed using either T-Test, Anova, Mann-Whitney or Kruskal-Wallis (SPSS). Multivariate analysis (One way Permanova) used to identify significant differences between fungal abundance datasets. Soil fungal community compositions were visualised using detrended correspondence analysis (DCA) based on Bray-Curtis similarity index after data was box-cox transformed and all zero rows / columns, missing rows / columns and singleton rows / columns removed. Diversity indices (H and E_H) were analysed after data had been rarefied, by calculating the proportional OTU composition (number of reads of an OTU in a sample/total number of reads in a sample). This method was used to remove any variability in sequence read numbers between the chips. All results were considered significant at the $p < 0.05$ level.

7.5. Results

Four 316 Ion Torrent™ Chips (each with a stated capacity of 2-3 million reads per chip; 300-1000 Mbp; https://www.lifetechnologies.com/order/catalog/product/4483324) were used to sequence all sample data. For both sites a total of 168 sampling points were used (GA = 83 and MC = 85). There were no significant differences between the number of fungal amplicons per sample sequenced between the chips (23962 ± 1453 S.E. Kruskal-Wallis test; $X^2 = 1.1$, df = 3, $p = 0.80$). The number of fungal sequences per site varied from 583 to 34511 (mean 17400) for GA and 5529 to 61858 (mean 30370) for MC, as shown in Appendix 9.8.2, results from diversity analysis are unlikely to be quantitatively affected by differences in sequencing intensity between chips. An average of 372 OTUs, with 306 named taxa were identified. The majority of fungal sequences recovered belonged to the Ascomycota and Basidiomycota (52% and 33% respectively), 4.5% of fungal sequences were not identified to phylum level. Non-fungal eukaryotic (mostly Rhizaria, Virdiplantae or Animalia) totalled 15.9% (4524 sequences) were excluded from further analyses (See Appendix 9.8.2). Four samples exhibited disproportionately high levels of non-fungal reads, suggesting either contamination or sequencing error, so were removed from the analysis. A list of the 200 most common species found shown in Appendix 9.8.3.

7.5.1. Bio-inoculants (BI)

Each BI was independanlty sequenced ($n = 2$) and the total number of sequences (fungal / non-fungal), OTUs and phylum abundance (%) of each BI is given in Table 7.6. Biagro® S failed to sequence, the BI contained high levels of humics which could not be removed and may have interfered with the PCR process.

Table 7.6. Sequencing data of individual BIs. Number of sequences, operational taxanomic units (OTUs) and % phyla abundance

		Biagro® Grass	**Biagro® MP**	**Single MF inoculant**	**Biagro® Phos N**
No. sequences	*Fungi*	27639	5748	22689	17142
	Non-fungi	1792	238	1915	20
OTUs		315	152	532	98
Phylum (% abundance)					
Ascomycota		86.74	94.47	56.58	98.02
Basidiomycota		10.38	4.38	36.08	1.92
Chytridiomycota		0.00	0.66	0.07	0.01
Fungi incertae sedis		1.46	0.00	1.35	0.01
Glomeromycota		0.91	0.17	3.30	0.01

The most abundant fungal phyla sequenced across all the BIs was Ascomycota. Plant-beneficial fungi *Penicillium* sp., *Mortierella* sp. and *Aspergillus* sp. were some of the most abundant Ascomycota found within both Biagro® Grass and the Single MF inoculant (Appendix 9.8.11); Whilst Biagro® PhosN and Biagro® MP were mostly dominated by yeasts (Saccharomycetes) (Appendix 9.8.11). All BIs which claimed to contain MF were found to contain *Rhizophagus* sp.

7.5.2. Site (GA vs MG)

Multivariate analysis of the sequence data (plots which had no BI application, $n = 65$) found Site to be a significant factor on individual fungal taxa abundance (Permanova, $p = 0.0001$) (Fig. 7.6). The abundance of several fungal phyla differed significantly between sites (Table 7.7). Basidiomycota were more abundant at MG (T-test, $p < 0.001$), whereas Chytridomycota, Fungi incertae sedis and Glomermycota were more abundant at GA (T-test , $p < 0.001$). There were no significant differences in the diversity of species between the sites.

Table 7.7. Differences in abundance of fungal phyla (%) between sites, Gadlas (GA) and Morfa Ganol (MG). Asterisk is for groups where data were not normally distributed and a non-parametric statistical test used (Mann-Whitney). Where there was a significant treatment effect, different superscript letters indicate significantly different means (T-test and Mann-Whitney, $p < 0.05$). Shannon index (H) and equitability (E_H) are based on operational taxonomic unit counts (OTUs). Values in parenthesis are ± 1 standard deviation. Diversity measures based on rarefied dataset

	% Abundance	
Fungal phylum abundance (%)	**GA** (***n*** **= 33)**	**MG** (***n*** **= 32)**
Ascomycota	50.91 (± 13.54)	53.89 (± 16.39)
Basidiomycota	24.89[a] (± 9.24)	38.44[b] (± 16.85)
*Blastocladiomycota**	0.08 (± 0.18)	0.01 (± 0.02)
*Chytridiomycota**	2.83[a] (± 1.44)	1.00[b] (± 0.77)
*Fungi incertae sedis**	13.27[a] (± 7.82)	3.11[b] (± 2.34)
*Glomeromycota**	2.20[a] (± 1.57)	0.90[b] (± 0.61)
Diversity		
Shannon index (H)*	3.39 (± 0.45)	3.31 (± 0.46)
Equitability (E_H)*	0.77 (± 0.09)	0.74 (± 0.09)

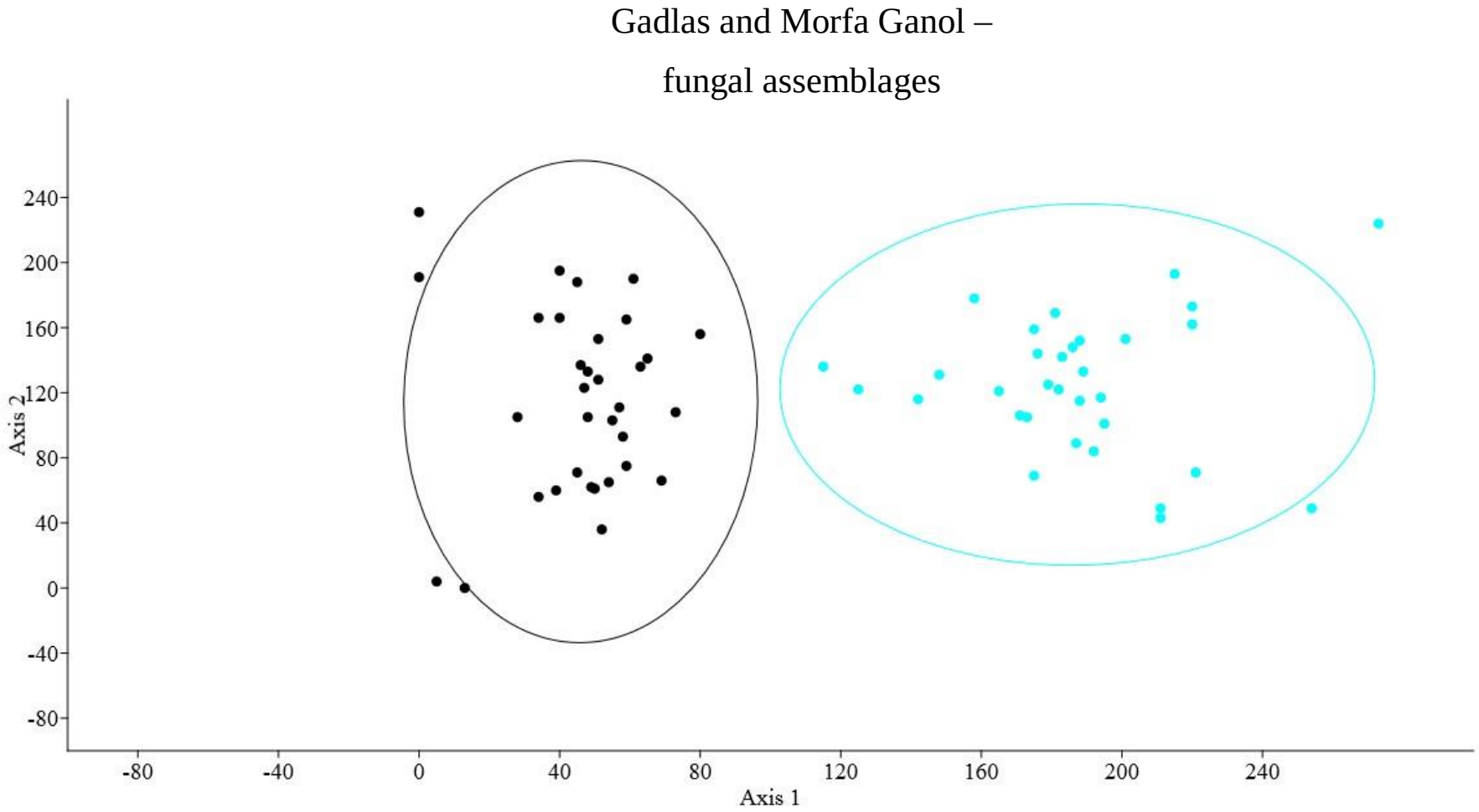


Figure 7.6. Plots of detrended correspondence analysis (DCA) of soil samples from two sites. Gadlas (GA) (●) and Morfa Ganol (MG) (●). Ellipses represent 95% confidence intervals. Eigen values for Axis 1 = 0.39, Axis 2 = 0.24

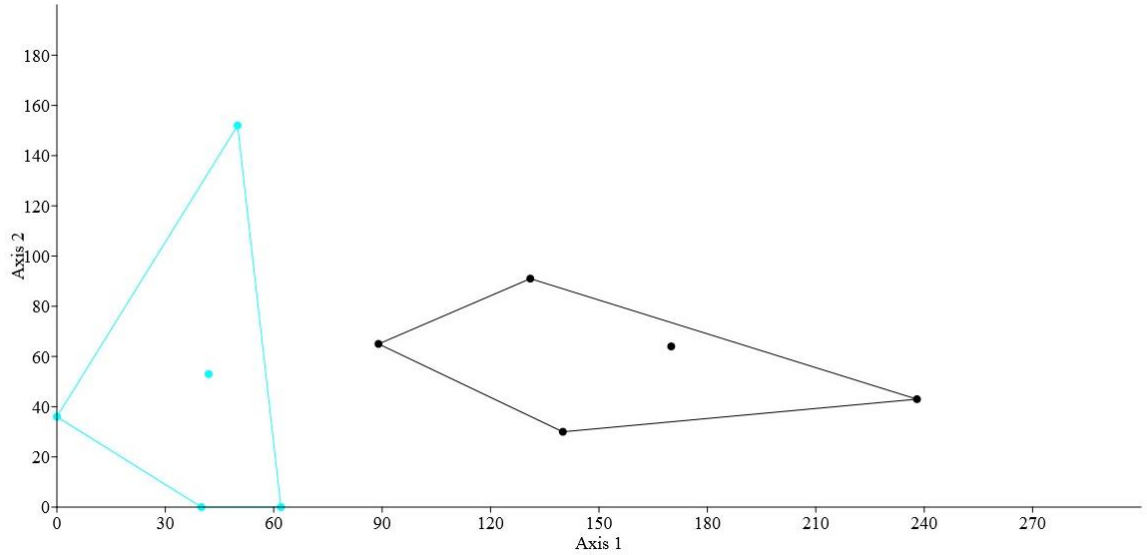
7.5.3. Sampling date

Sampling date was found to be a significant factor at both GA and MG on fungal taxa abundance (Permanova, $p = 0.002$ and 0.019 respectively), DCA plots of each site having clear temporal separations between fungal sequences (Fig. 7.7*a* and *b*). Within site analysis of fungal phyla found time to be a significant factor only at MG, with significant increases in September for two phyla, Ascomycota (T-test, $p = 0.04$) and Glomermycota (T-test, $p = 0.014$), and a significant reduction in Basidiomycota abundance (T-test, $p = 0.02$) (Table 7.8). Diversity measures were found not to differ significantly between sampling times at either site.

Table 7.8. Fungal phyla abundance (%) at two sampling dates (July and September 2013) of two Sites (GA and MG). Diversity measures, Shannon index (*H*) and Shannon Equitability (E_H), based on operational taxonomic unit counts (OTUs). Where there was a significant treatment effect, different superscript letters indicate significantly different means within each site (T-test, $p < 0.05$). Values in parenthesis are ± 1 standard deviation

	GA		**MG**	
Fungal phyla abundance (%)	**July** (***n*** **= 5)**	**September** (***n*** **= 5)**	**July** (***n*** **= 5)**	**September** (***n*** **= 3)**
Ascomycota	52.85 (± 13.85)	54.68 (± 14.52)	42.68^{a} (± 10.89)	66.82^{b} (± 15.57)
Basidiomycota	24.19 (± 8.38)	24.42 (± 12.08)	51.09^{a} (± 9.53)	17.55^{b} (± 7.16)
Blasocladiomycota	0.10 (± 0.23)	0.00	0.01 (± 0.01)	0.03 (± 0.02)
Chytridiomycota	2.38 (± 1.51)	2.01 (± 1.06)	0.55 (± 0.30)	2.00 (± 1.65)
Fungi incertae sedis	13.05 (± 7.96)	11.96 (± 7.01)	3.13 (± 2.47)	5.71 (± 3.36)
Glomeromycota	3.37 (± 1.54)	2.95 (± 0.64)	0.20^{a} (± 0.07)	1.57^{b} (± 1.28)
Diversity				
Shannon index (H)	3.20 (± 0.73)	3.16 (± 0.45)	3.42 (± 0.36)	3.59 (± 0.33)
Shannon equitability (E_H)	0.73 (± 0.15)	0.74 (± 0.05)	0.75 (± 0.05)	0.77 (± 0.05)

a. Gadlas (GA) site



b. Morfa Ganol (MG) site

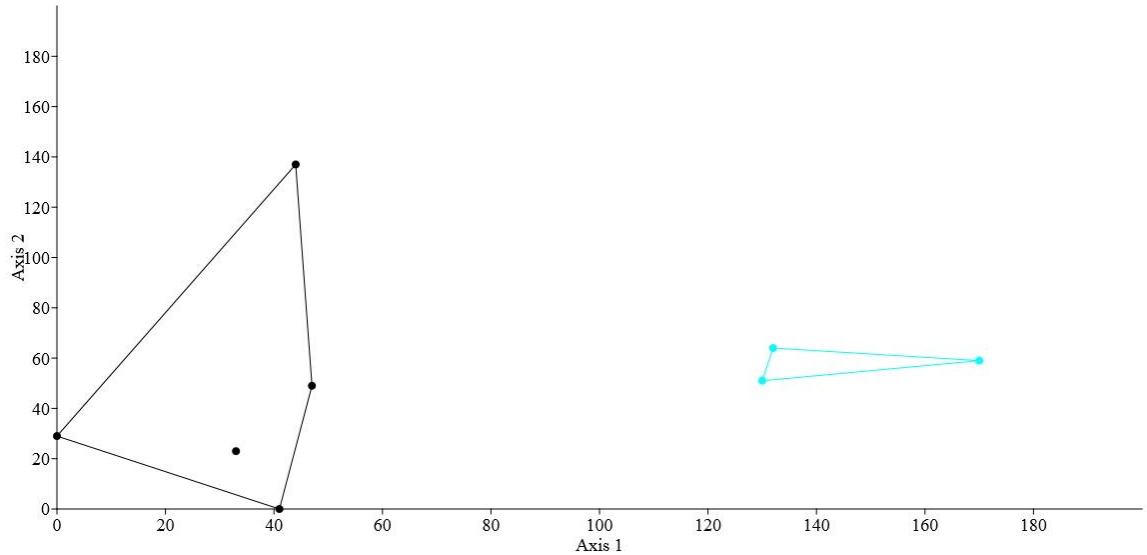


Figure 7.7 Plots of detrended correspondence analysis (DCA) of control plots from both sites, ***a***: GA, Eigen values of 0.35 and 0.15 (Axis 1 and 2 respectively) and ***b***: MG, Eigen values of 0.25 and 0.13 (Axis 1 and 2 respectively). Samples taken at two sampling dates, July (●) and September (●) 2013

Examining the most abundant species at each site, an example of the output is given in Table 7.9, revealed variations in species abundance within individual phyla. For example, Ascomycota species such as *Thelebolus* sp. was more abundant in July at GA, 15.0% (± 24.0) compared to 1.7% (± 1.7) in September, but was not significant, whilst *Pyrenochaeta* sp. and *Didymella* sp. were both significantly more abundant in September (15.3% (± 8.7) and 7.3% (± 4.9) respectively) than July (2.6% (± 2.0) and 1.1% (± 0.8), respectively) (T-test, $p < 0.05$) (Table 7.8).

Basidiomycota, *Conocybe* sp. and *Coprinopsis* sp. were ~ two fold more abundant in July than September at MG (Appendix 9.8.4), although not significant. Ascomycota, *Veronaea* sp. and *Penicillium* sp. were the only two taxa to be significantly different between sampling dates at MC, both with higher abundance in September, 16.1% (± 4.5) and 7.9% (± 2.8) respectively, than July, 9.9% (± 1.9) and 2.8% (± 1.1) respectively (T-test, $p < 0.05$) (Appendix 9.8.4).

Table 7.9. GA site samples taken in July (n = 5) and September (n = 5) 2013. Showing the top 19 fungal sequences and the mean, median (med), maximum (max) and minimum (min) relative abundance (%) of the Ion Torrent™ output. Totals for each phylum and % of sequences identified to family and genus. Asterisk indicates significant difference between sampling dates (T-test, $p < 0.05$)

					July				Sept			
Phylum	**Class**	**Order**	**Family**	**Genus**	***mean***	***med.***	***max.***	***min.***	***mean***	***med.***	***max.***	***min.***
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	12.18	10.30	25.60	3.95	11.10	7.07	25.60	3.95
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Pyrenochaeta*	2.61	1.87	5.77	0.74	9.52	5.77	29.79	0.74
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	15.01	3.32	56.76	0.27	7.74	1.21	56.76	0.27
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	6.11	5.83	10.87	3.50	6.36	4.44	18.84	0.92
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella*	1.10	1.03	2.45	0.32	4.48	2.45	13.15	0.32
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 55	4.58	2.89	15.84	0.00	4.38	1.87	18.22	0.00
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	3.95	4.47	7.50	1.37	3.39	3.11	7.50	1.21
Ascomycota	Leotiomycetes	Helotiales	X	OTU 16	0.29	0.26	0.69	0.00	2.33	0.59	12.87	0.00
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Piriformospora	1.79	0.81	4.28	0.00	1.81	0.81	4.94	0.00
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	OTU 19	0.61	0.27	2.23	0.00	1.77	1.58	5.19	0.00
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OTU 3238	0.00	0.00	0.00	0.00	1.77	0.00	16.67	0.00
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 69	0.49	0.00	2.45	0.00	1.68	0.21	7.57	0.00
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OTU 9	1.83	0.58	4.92	0.00	1.55	1.05	4.92	0.00
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Waitea	3.09	2.10	7.50	0.34	1.44	0.34	7.50	0.00
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 2612	2.91	0.00	12.69	0.00	1.34	0.00	12.69	0.00
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 17	1.64	1.63	3.11	0.53	1.24	1.03	3.11	0.25
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Mollisia	2.05	0.50	9.09	0.00	1.20	0.50	9.09	0.00
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	1.80	1.22	5.15	0.13	1.10	0.57	5.15	0.13
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	1.22	1.76	2.02	0.00	1.03	1.16	2.02	0.00
		Totals			***mean***	***med.***	***max.***	***min.***	***mean***	***med.***	***max.***	***min.***
		% Fungi id. to family			79.8	78.6	92.1	70.8	75.10	75.48	85.88	65.30
		% Fungi id. to genus			72.4	71.0	90.0	59.5	64.08	64.25	68.35	59.69
		Ascomycota			52.9	44.9	70.5	40.1	54.68	60.03	70.51	36.23
		Basidiomycota			24.2	22.7	32.2	12.1	24.42	27.05	38.84	8.69
		Blastocladiomycota			0.1	0.0	0.5	0.0	0.00	0.00	0.00	0.00
		Chytridiomycota			2.4	3.2	3.8	0.3	2.01	2.08	3.03	0.50
		Fungi incertae sedis			13.1	10.8	26.0	5.5	11.96	7.02	19.75	6.60
		Glomeromycota			3.4	3.3	5.3	1.2	2.95	3.05	3.81	2.21
		Not identified			4.0	3.8	5.8	3.0	3.96	3.19	5.93	2.61

7.5.4. Inorganic nitrogen application

7.5.4.1. Effect of N on fungi one week after application

Control plots of GA site were sampled in June 2013 (Fig. 7.2 section 7.4.1.1) and one week later, after the application of 25 kg ha^{-1} NH_4NO_3. Multivariate analysis of the sequence data found a significant effect of nitrogen application on individual fungal taxa (Permanova, p = 0.0019). DCA plots clearly show a separation between plots receiving nitrogen (N) fertiliser and those receiving no N (Fig. 7.8). Ascomycota and Fungi incertae sedis were the only phyla to be significantly affected by N application (Table 7.10). N application increasing Ascomycota abundance and reducing Fungi incertae sedis (T-test, p = 0.02 and 0.007 respectively).

Table 7.10. Effects of nitrogen (N) treatment, before (- N) and after (+ N) (25 kg ha^{-1}) on abundances of fungal phyla (%) and Diversity measures of Shannon index (H) and Shannon Equitability (E_H) (based on operational taxonomic unit counts (OTUs)). Where there was a significant treatment effect, different superscript letters indicate significantly different means (T-test, $p < 0.05$). Values in parenthesis are ± 1 standard deviation

	Nitrogen treatment	
Fungal phyla abundance (%)	**- N (*n* = 5)**	**+ N (*n* = 6)**
Ascomycota	35.51[a] (± 15.20)	55.25[b] (± 7.35)
Basidiomycota	25.80 (± 10.41)	19.44 (± 4.28)
Blastocladiomycota	0.03 (± 0.04)	0.21 (± 0.28)
Chytridiomycota	4.10 (± 2.26)	2.54 (± 0.83)
Fungi incertae sedis	25.97[a] (± 4.99)	14.13[b] (± 6.04)
Glomeromycota	1.39 (± 1.02)	0.88 (± 0.63)
Diversity		
Shannon index (H)	3.05[a] (± 0.40)	3.52[b] (± 0.13)
Shannon equitability (E_H)	0.68[a] (± 0.06)	0.81[b] (± 0.03)

Analysis of the most abundant taxa found some species variation between treatments. There was a significant reduction in the relative abundance of *Mortierella* sp. after N application, from 25.2% to 1.9%. *Tricladium* sp. and *Gamsiella* sp. both increased in abundance following N application from 4.3% and 0.3%, to 14.1% and 10.4% respectively (Table 7.11). N application increased fungal diversity (H) (T-test, p = 0.021) and equitability (E_H) amongst the species (T-test, p = 0.002) (Table 7.10).

Table 7.11. Individual taxa showing significant changes with treatment (T-test and Mann-Whitney, $p < 0.05$). Mean abundance (%) from plots sampled in June 2013 before (- N) ($n = 5$) and after (+ N) ($n = 6$) application. Values in parenthesis are ± 1 standard deviation. Top 200 taxa shown in Appendix 9.8.5

			Total (%)	
Phylum	**Family**	**Genus**	**- N**	**+ N**
Fungi incertae sedis	Mortierellaceae	*Mortierella*	25.2 (± 5.0)	1.9 (± 0.7)
Ascomycota	Helotiaceae	*Tricladium*	4.3 (± 1.7)	14.1 (± 4.0)
Fungi incertae sedis	Mortierellaceae	*Gamsiella*	0.3 (± 0.2)	10.4 (± 5.1)
Ascomycota	Pleosporaceae	*Pyrenochaeta*	0.3 (± 0.1)	1.7 (± 1.3)
Ascomycota	Didymellaceae	*Didymella*	0.2 (± 0.3)	1.0 (± 0.4)
Chytridiomycota	Alphamycetaceae	*Betamyces*	2.0 (± 0.7)	0.7 (± 0.2)
Basidiomycota	Tremellaceae	*Cryptococcus*	0.7 (± 0.3)	1.7 (± 0.9)

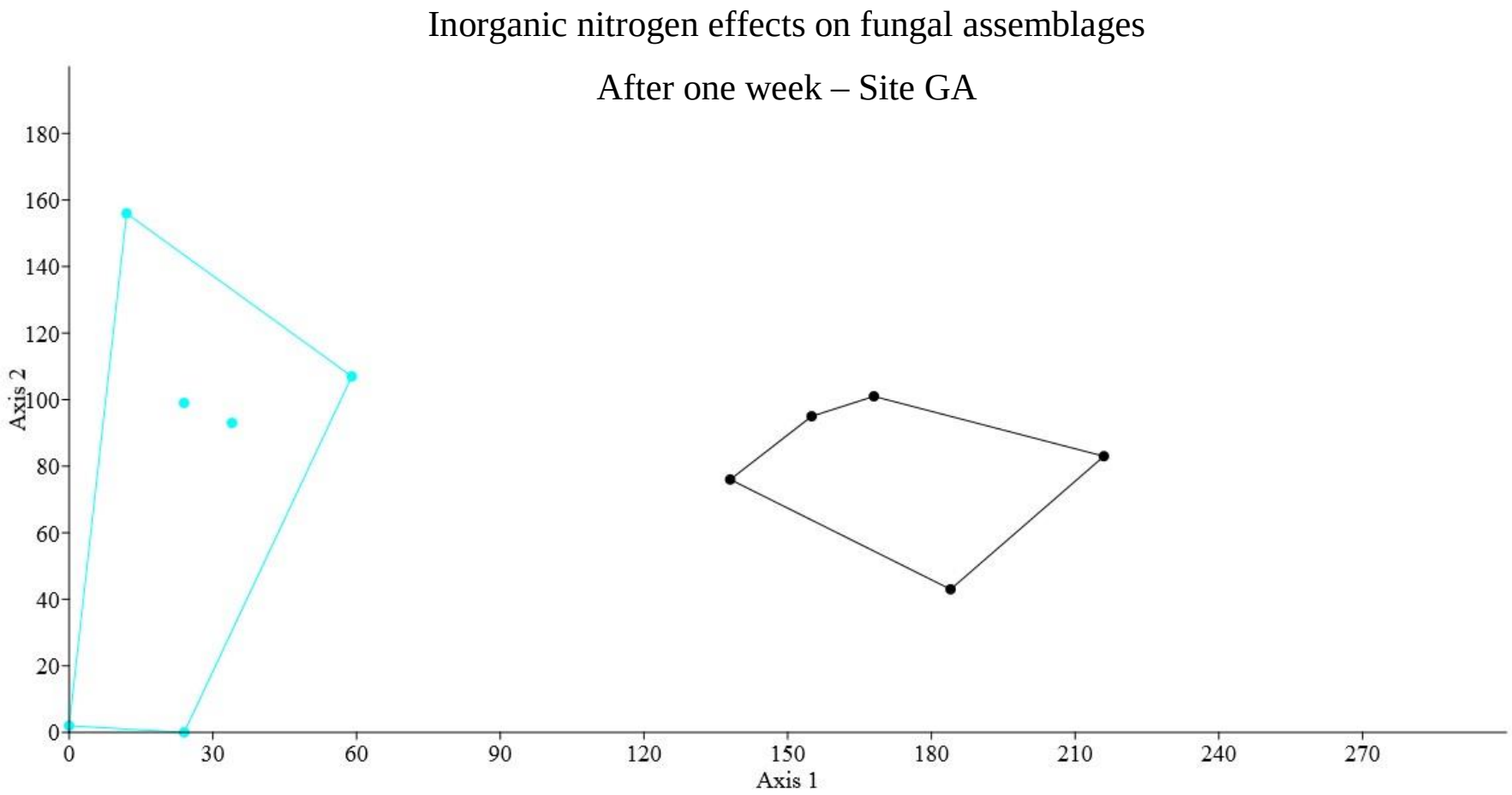


Figure 7.8. Plots of detrended correspondence analysis (DCA) of GA plots before N application (●) and 7 days later (●) taken in June 2013. Eigen values of 0.39 (Axis 1) and 0.16 (Axis 2)

7.5.4.2. Analysis of N addition on fungi for July and September - GA

Using the no addition buffer plots (Fig 7.2, Section 7.4.1.1) and control plots of GA which received 50 kg ha^{-1} NH_4NO_3 (June) and a further 50 kg ha^{-1} (July). Multivariate analysis of the sequence data found a significant effect of nitrogen application on individual fungal taxa of soil samples (Permanova, p = 0.0001). DCA plots clearly show a separation between plots receiving nitrogen and those receiving none (Fig. 7.9). Univariate analysis (Nitrogen and sampling date as factors) found N application to be a significant factor, with an increase in both fungal diversity (H, p = 0.016) and species evenness (E_H, p = 0.018) (Table 7.12). There was no significant effect of nitrogen application on fungal phyla abundances. Basidiomycota did increase in abundance after N application in July, but this was not repeated in September, univariate analysis showed time to be the significant factor (p = 0.051) and that there was a significant interaction between both factors (Nitrogen application*Time, p = 0.048), see also Table. 7.8 (Sampling date, Section 7.5.3).

Table 7.12. Effects of nitrogen application (+ N) and no application (- N) on fungal phyla abundance (%) at two sampling dates (July and September 2013) at GA. Diversity measures, Shannon index (H) and Shannon Equitability (E_H), based on operational taxonomic unit counts (OTUs). Where there was a significant treatment effect, different superscript letters indicate significantly different means (T-test, Mann-Whitney, $p < 0.05$). Values in parenthesis are ± 1 standard deviation

	July		**September**	
Fungal phyla abundance (%)	**- N (n = 5)**	**+ N (n = 6)**	**- N (n = 6)**	**+ N (n = 5)**
Ascomycota	52.85 (± 13.85)	45.61 (± 7.28)	56.59 (± 13.81)	58.72 (± 12.53)
Basidiomycota	24.19 (± 8.38)	34.99 (± 7.22)	24.32 (± 10.81)	19.80 (± 6.18)
Blasocladiomycota	0.10 (± 0.23)	0.11 (± 0.23)	0.00	0.01 (± 0.03)
Chytridiomycota	2.38 (± 1.51)	3.33 (± 1.19)	1.91 (± 0.98)	2.87 (± 1.14)
Fungi incertae sedis	13.05 (± 7.96)	8.00 (± 3.17)	11.08 (± 6.63)	8.75 (± 3.22)
Glomeromycota	3.37 (± 1.54)	1.62 (± 0.71)	2.60 (± 1.03)	3.64 (± 2.30)
Diversity				
Shannon index (H)	3.21^a (± 0.73)	3.72^b (± 0.17)	3.16^a (± 0.45)	3.61^b (± 0.31)
Shannon equitability (E_H)	0.73^a (± 0.15)	0.83^b (± 0.03)	0.74^a (± 0.05)	0.82^b (± 0.05)

Analysis of the most abundant taxa found some species variation between treatments. Table 7.13 highlight some of the significant changes in taxa at both sampling dates. *Mortierella* sp.

and *Waitea* sp. were both significantly reduced with N application, whilst *Didymella* sp., *Cladosporium* sp. and *Gamisella* sp. all significantly increased in abundance after N application in July (Table 7.13). September similarly saw reduced abundance of *Mortierella* sp., but also *Didymella* sp., *Gamsiella* sp. and *Veronaea* sp. increased in abundance for the N-treated plots (Table 7.13).

Table 7.13. Mean relative abundance (%) of taxa from plots sampled in July / September 2013 before (- N) and after (+ N) application at GA. 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 (T-test, Mann-Whitney, $p < 0.05$). Top 200 taxa shown in Appendix 9.8.6 and 9.8.7

			July		**September**	
Phylum	**Family**	**Genus**	**- N (*n* = 5)**	**+ N (*n* = 5)**	**- N (*n* = 6)**	**+ N (*n* = 5)**
Ascomycota	Thelebolaceae	*Thelebolus*	15.0 (± 24.0)	0.8 (± 0.4)	1.68 (± 1.7)	3.25 (± 3.5)
Fungi incertae sedis	Mortierellaceae	*Mortierella*	12.2^{a} (± 8.4)	2.8^{b} (± 2.3)	10.2^{a} (± 6.7)	2.5^{b} (± 0.9)
Ascomycota	Helotiaceae	*Tricladium*	6.1 (± 2.8)	7.4 (± 3.5)	6.6 (± 7.2)	7.2 (± 3.4)
Ascomycota	Pleosporaceae	*Pyrenochaeta*	2.6 (± 2.0)	5.0 (± 2.3)	15.28 (± 8.73)	8.4 (± 11.5)
Ascomycota	Herpotrichiellaceae	*Veronaea*	3.9 (± 2.5)	2.8 (± 1.0)	2.9^{a} (± 1.5)	4.8^{b} (± 1.2)
Ascomycota	Didymellaceae	*Didymella*	1.1^{a} (± 0.8)	4.5^{b} (± 2.5)	7.3^{a} (± 4.9)	1.6^{b} (± 0.5)
Ascomycota	Davidiellaceae	*Cladosporium*	0.5^{a} (± 0.4)	4.8^{b} (± 1.9)	1.1 (± 0.6)	2.3 (± 2.2)
Fungi incertae sedis	Mortierellaceae	*Gamsiella*	0.1^{a} (± 0.1)	2.9^{b} (± 1.0)	0.03^{a} (± 0.03)	4.8^{b} (± 3.2)
Basidiomycota	Ceratobasidiaceae	*Waitea*	3.1^{a} (± 2.9)	0.3^{b} (± 0.3)	0.07 (± 0.16)	0.01 (± 0.02)

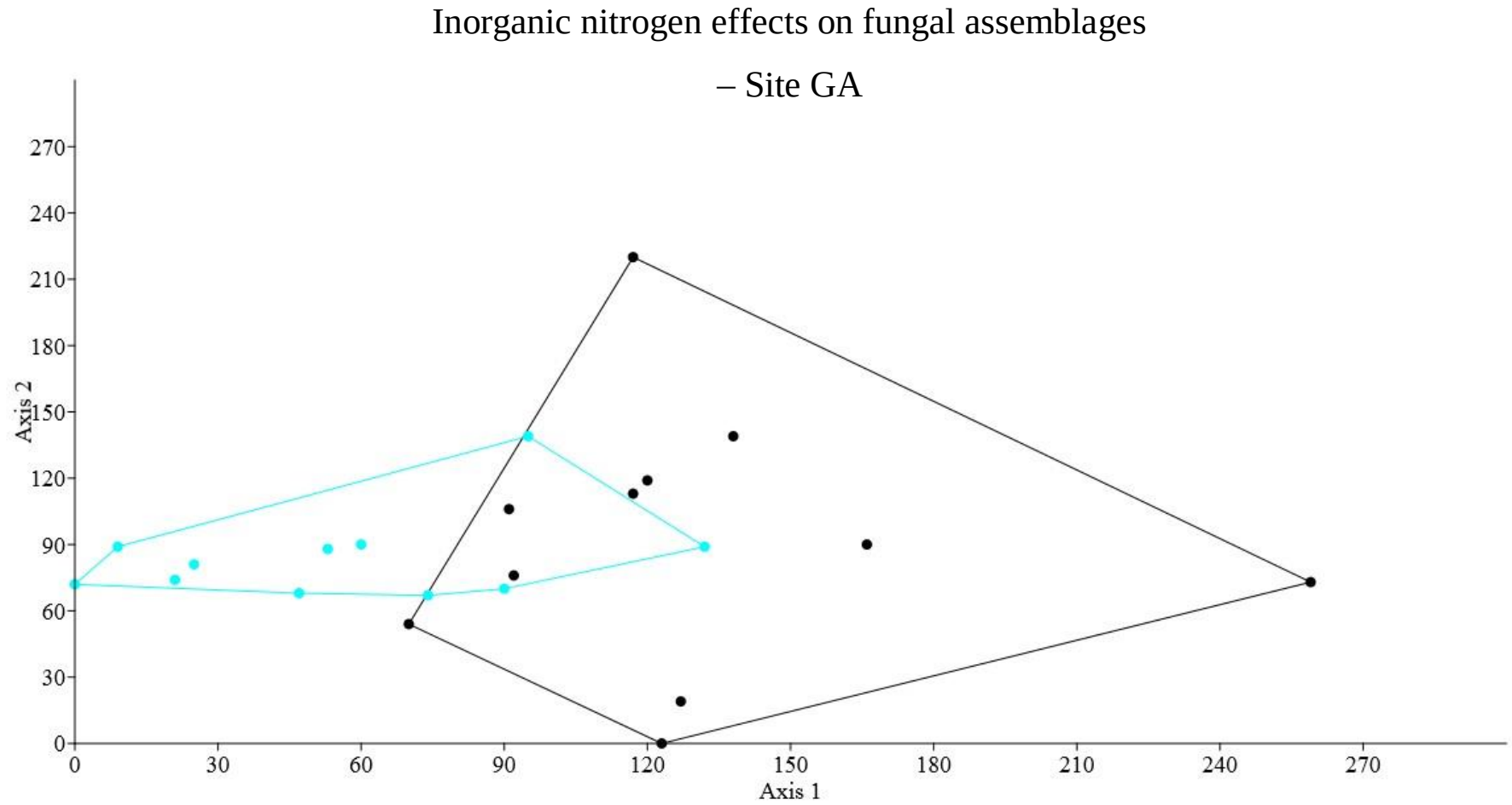


Figure 7.9. Plots of detrended correspondence analysis (DCA) of sequence data of plots from GA (July and September 2013). Inorganic N applied (●) (June (50 kg ha^{-1}) and July (50 kg ha^{-1})), no N applied (●). Eigen values of 0.33 (Axis 1) and 0.22 (Axis 2)

7.5.5. Effect of phosphate on fungi

Fungal DNA sequences, extracted from soil samples of both the P-index trial (MG*a*, Fig. 7.3) and inorganic phosphate fertiliser trial (MG*b*, Fig. 7.4) taken in August 2014, were analysed to assess the impact of phosphate on fungal community assemblages.

7.5.5.1. P-index trial - MGa

Multivariate permanova analysis of soil samples taken in August 2014, found no significant differences between control and BI-treated grass plots in relative fungal taxa abundance. Phyla abundance and diversity measures were simillarlly unaffected by either factor, BI application or P-index (appendix 9.8.8).

The only significant result was with regards to a *Rhizophagus* sp., which was found to be statistically more abundant (Mann-Whitney, $p = 0.014$) in BG-treated plots (0.44% ± 0.1 SEM, $n = 9$) than control plots (0.21% ± 0.03 SEM, $n = 9$). There was also a negative correlation of *Rhizophagus* sp. with soil phosphate for the BG-treated grass plots (Spearman's rho = -0.700, $p = 0.036$) (Fig.7.10), the low r^2 value (0.08) however an indication that there are other factors contributing to the correlation.

7.5.5.2. Inorganic fertilisers (TSP and RP) – MGb

Oneway Permanova analysis of the fungal sequence data found no significant treatment effect of the inorganic fertilisers (applied March 2014), triple super phosphate (TSP) and rock phosphate (RP) (100 kg ha^{-1} P_2O_5) on relative fungal taxa abundance. Fungal phyla and diversity measures were also found not to differ significantly between P treatments. There was also no interactive effects between P treatment and BI application.

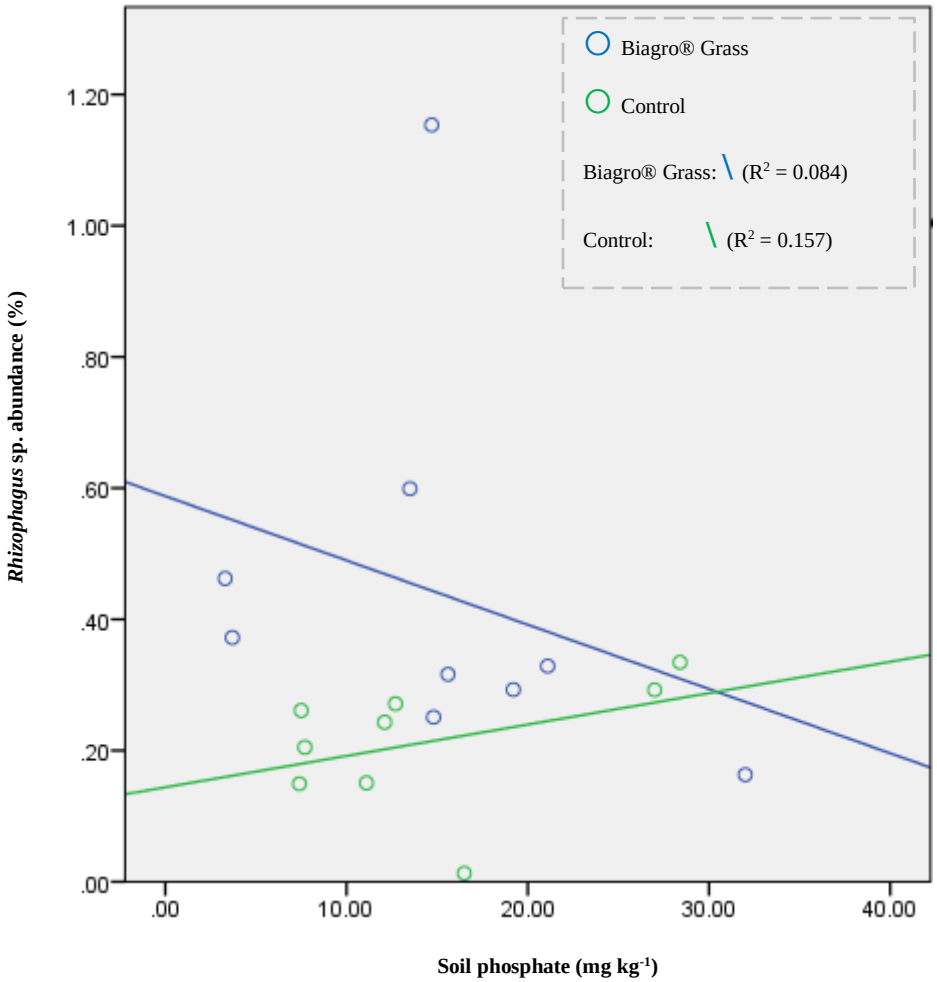


Figure 7.10. Mean relative abundances (%) of *Rhizophagus* sp. (y-axis) with soil phosphate (mg kg^{-1}) (x-axis) of BG-treated grass and control plots (n = 9) at MG*a*. Trend lines of each treatment shown (R^2 values shown), BG plots having a negative correlation between *Rhizophagus* sp. abundance and soil phosphate (Spearman's rho = -0.700, p = 0.036)

7.5.6. BI application

7.5.6.1. Gadlas (GA)

Multivariate analysis of sequences from soil samples taken from GA plots (Fig. 7.2, Section 7.4.1.1) in June 2013, prior to any inorganic N fertiliser application but ten months after BI application, two way permanova (BI and application rate) found a significant effect of both BI application (p = 0.005) and application rate (p = 0.03) on fungal OTU abundances. Pairwise comparisons found a significant difference between control plots and BG-treated plots at ten times application rate (p = 0.05) (Table 7.14*b*). BG-treated grass at recommended application rates was relatively close to being significantly different to controls with a p value of 0.07 (Table 7.14*a*).

Table 7.14*a*. Probability values of pairwise comparisons of bio-inoculants at Gadlas (GA) at recommended application rates (n = 3). Biagro® MP not featured due to only two replicates available

	Control	**Biagro® Grass**	**Biagro® MP**	**Biagro® S**
Control	-	0.07	n/a	0.68
Biagro® Grass	0.07	-	n/a	0.50
Biagro® MP	n/a	n/a	-	n/a
Biagro® S	0.68	0.50	n/a	-

* result considered significant at the 0.05 level

Table 7.14*b*. Probability values of pairwise comparisons of bio-inoculants at Gadlas (GA) at ten times application rates (n = 3)

	Control	**Biagro® Grass**	**Biagro® MP**	**Biagro® S**
Control	-	0.05*	0.14	0.68
Biagro® Grass	0.05*	-	0.41	0.60
Biagro® MP	0.14	0.41	-	0.41
Biagro® S	0.68	0.60	0.41	-

Analysis of the most abundant taxa found there was a significant increase for *Tricladium* sp. in BG-treated plots (Anova, p = 0.007) (Table 7.15). Analysis of fungal phyla and diversity measures, however revealed no significant effect of BI application or application rate (Appendix 9.8.9).

Table 7.15. Mean abundance (%) of taxa from GA plots sampled in June 2013 treated with BIs. Values in parenthesis are ± 1 standard deviation. Where there was a significant treatment effect, different superscript letters indicate significantly different means (Anova, $p < 0.05$)

	BI			
Genus	***Control*** **(*n* = 5)**	***BG*** **(*n* = 6)**	***BMP*** **(*n* = 5)**	***BS*** **(*n* = 6)**
Mortierella	25.24 (± 8.05)	15.83 (± 3.21)	15.90 (± 12.94)	21.16 (± 5.02)
Tricladium	4.26[a] (± 1.66)	13.04[b] (± 8.17)	3.49[a] (± 2.38)	4.22[a] (± 1.61)
Veronaea	6.77 (± 3.36)	5.81 (± 2.75)	3.20 (± 1.51)	6.20 (± 5.58)
Thelebolus	4.05 (± 2.82)	3.88 (± 7.16)	11.65 (± 12.91)	3.07 (± 2.93)
Preussia	1.41 (± 0.67)	1.45 (± 1.32)	12.37 (± 16.91)	1.05 (± 0.45)
Piriformospora	0.22 (± 0.27)	0.24 (± 0.36)	4.52 (± 5.32)	1.10 (± 1.43)

7.5.6.2. Morfa Ganol (MG)

Analysis of sequences from soil samples taken from MG*b* (Fig. 7.4, Section 7.4.1.2.2) plots in August 2014, after the application of nitrogen fertilisers and phosphate treatments, saw a significant effect of BI application on fungal OTU abundances between plots (Permanova, p = 0.012), pairwise comparisons, shown in Table 7.16, indicate a significant effect of PN and SSI application on the relative abundance of fungal OTUs. There were no significant differences observed between phyla or diversity measures for BI-treated plots compared to control plots (Appendix 9.8.10)

Table 7.16. Probability values of pairwise comparisons of bio-inoculants at MG*b* (n = 9)

	BG	**Control**	**PN**	**SSI**
BG	-	0.3131	0.0128*	0.2526
Control	0.3131	-	0.0012*	0.017*
PN	0.0128*	0.0012*	-	0.2425
SSI	0.2526	0.017*	0.2425	-

* result considered significant at the 0.05 level

Analysis of the most abundant taxa found some species variation between BI-treated plots (Table 7.17). Abundances of *Veronaea* sp. were significantly higher in BI-treated plots (Anova, p = 0.038), whilst PN-treated plots exhibited higher abundance of *Pyrenochaeta* sp. (Anova, p = 0.009). *Coprinopsis* sp. was significantly reduced in abundance within SSI and PN-treated plots (Kruskal-Wallis, p = 0.012).

Table 7.17. Mean abundance (%) of taxa from plots sampled in August 2014 treated with BIs at MG*b*. Values in parenthesis are ± 1 standard deviation. Where there was a significant treatment effect, different superscript letters indicate significantly different means (Anova and Kruskal-Wallis, $p < 0.05$)

		BI		
Genus	***Control*** **(*n* = 9)**	***BG*** **(*n* = 9)**	***SSI*** **(*n* =9)**	***PN*** **(*n* = 9)**
Veronaea	9.81^{a} (± 5.13)	17.80^{b} (± 9.84)	18.27^{b} (± 4.63)	17.38^{b} (± 6.25)
Pyrenochaeta	0.78^{a} (± 0.61)	1.01^{a} (± 0.60)	0.91^{a} (± 0.45)	1.88^{b} (± 1.01)
Coprinopsis	11.56^{a} (± 18.87)	10.26^{a} (± 18.32)	0.87^{b} (± 1.18)	0.95^{b} (± 0.79)

7.6. Discussion

The Ion Torrent™ NGS platform utilised within the study was able to discern clear differences in fungal abundances of soils under varying treatments. Over 1 million reads were generated per chip sequenced (4,063,882 total of four chips). The number of reads per sample was ~23 000, and is in line with other studies which have utilised the same NGS platform (Brown et al. 2013). There was a diverse fungal community of 400 fungal OTUs across both sites. Fungal assemblages were found to be significantly affected by both treatment (BI application and inorganic nitrogen) location and sampling date.

Sequencing of the BI treatments revealed them to contain species detailed by the manufacturer, notably MF such as *Rhizophagus irregularis* (phylum: Glomermycota). However there was a surprisingly large amount of other micro-organisms also sequenced, for example 532 fungal OTUs were sequenced from the Single MF species inoculuant with around ~8 % of sequences being non-fungal; and whilst all the fungal BIs were found to contain many species that are known to be plant-beneficial organisms, *Penicillium* sp, *Trichoderma* sp. and *Mortierella* sp. for example (Appendix 9.8.11); does raise concerns as to the purity of BI products and the manufacturing process and may have future implications in light of expected regulatory changes regards BI registration and sale. The trap culture method employed to manufacture both the SSI and BG inoculants can result in ~ 10^3 backround micro-organisms per cm^{-3} BI (INVAM, personal comm.).

Gadlas (GA) soil, a brown earth loam, had higher abundances of several fungal phyla, Chytridomycota, Fungi incerate sedis and Glomermycota than Morfa Ganol (MG). GA soil was found to be higher in plant-available NO_3, NH_4 and PO_4 than MG soil, and soil nutrient status has been shown to be closely associated with fungal community composition (Allison, Hanson & Treseder 2007, Lauber et al. 2008). Nutrient availability effects may have been further exacerbated due to tillage, which has been shown to increase available nutrients (Sheng et al. 2013), and thus further impact on fungal communities; for example, MF belonging to the phylum, Glomermycota, increased in abundance within plant roots post tillage (Alguacil et al. 2008).

Plant nutrient availability also has a major effect on plant root morphology and consequently root exudate production, as such plants are able to regulate fungal community composition

(Shaw, Burns 2005, Nannipieri et al. 2008, Bainard et al. 2014). Rhizo-deposition can represent 10 to 40% of all the carbon assimilated by plants and is released into the soil in the form of carbon containing root secretions which is a substrate for soil micro-organisms (Lynch, Whipps 1990, Broeckling et al. 2008). The application of inorganic fertilisers, which increase plant nutrients, could potentially alter fungal community composition; whilst abiotic factors such as temperature have also been shown to affect root morphology and as such there will be seasonal variations in rhizo-deposition (Macduff et al. 1986, Poerwanto, Inoue & Kataoka 1989, Dehaghi, Sanavy 2003).

Fungal community composition has been shown to be a sensitive indicator of abiotic change (Kaisermann et al. 2015); for example fungal diversity indices have been shown to be susceptible to temporal changes, as with and other studies, with fungal taxa showing temporal shifts in dominance (Girvan et al. 2004, Dumbrell et al. 2011). Within their study, Dumbrell et al 2011 found seasonal variations in Glomermycota abundance for example. Within this study, MG saw increased abundance of the phyla Glomermycota and Ascomycota in September, and a reduction in Basidiomycota, with taxa such as *Penicillium* sp. (phylum Ascomycota; order Eurotiales) being more prevalent in September than July. *Penicillium* sp. is a ubiquitous soil fungi which prefers cool and moderate climates (Redman et al. 1999), commonly present wherever organic material is available. Similarly at the GA site, the genus *Pyrenochaeta* sp. and *Didymella* sp., (phyla Ascomycota; order Pleosporales) were found to be more prevalent in September than July, and are known to be saprobes of decaying matter (Câmara et al. 2002). There was potentially increased leaf litter at the Gadlas site, the plots were located near a wooded area. Furthermore, during this period there were no fertilisers applied to either site and resource availability can impact on intraspecific competition and resource partitioning within fungal communities (Tokeshi 1999, May, Crawley & Sugihara 2007). The sandy soil of MG for example was found to be low in available nutrients, such as nitrogen and phosphate, at seeding, and, may have resulted in increased resource competition as nutrients were depleted further with plant growth between July and September 2013.

This study examined the application of a nitrogen fertiliser (NH_4NO_3) and two phosphate fertilisers (TSP and RP). Of the inorganic fertilisers, ammonium nitrate was the only one to have a significant effect on fungal relative abundance, with both diversity and equitability indices increasing after N application. There are mixed reports on the effects of fertilisation on microbial species abundance and richness with both increases and decreases reported (Girvan et al. 2004, Allison, Hanson & Treseder 2007, Alguacil et al. 2008, Dumbrell et al. 2010, Lin

et al. 2012, Paungfoo-Lonhienne et al. 2015). For example, *Mortierella* sp. (phylum Fungi incerate sedis; order Mortierellales) abundance at the GA site 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, Bååth & Söderström 1990), the authors ascribing the fungal changes to changes in pH. Several studies have shown how pH is the main driver of micro-organism community change (Baath, Lundgren & Soderstrom 1984, Baath, Arnebrant 1993, Rousk et al. 2010).

Prolonged applications of inorganic fertilisers, however, have been shown to have significant effects on soil fungal communities (Arnebrant, Bååth & Söderström 1990). The converging convex hulls, seen on the DCA plots of fungal communities within this study, would suggest a transient response to effects of applied inorganic N (Girvan et al. 2004). For example after the application of N in July Basidiomycota abundance increased at the GA site, by September the abundance had returned to similar levels as plots which had received no N.

Phosphate effects appeared to have less of an impact on fungal assemblages within this study. Growth responses of the grass were also found to be unresponsive to phosphate application (Chapter 6). 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 the application of NH_4NO_3 and PO_4 treatments may have reduced the effectiveness of the applied fertilisers. Soil phosphate level i.e. plant-available P was also found to have very little effect on the relative abundance of fungi within the soil. There were no discernible differences between overall fungal communities of either Biagro® Grass-treated or control plots (MG*a* site) with varying levels of plant-available P. The P-index is a measure of the plant-available P fraction, and not total P (which did not differ largely between plots (Section 6.4.3.1)), fungi therefore may not have been P-limited in the lower range, as the index might imply, and were largely unaffected by the variable plant-avaialble P fraction. The results appear to suggest that fungal communities within the tested soils of this study, where less affected by phosphate availability.

Many studies of microbial changes in the field focus on fungi within plant roots and the plant root rhizosphere (Santos-Gonzalez, Finlay & Tehler 2007, Dumbrell et al. 2011), the dynamic nature of the root rhizosphere impacting on micro-organisms. Non-rhizospheric soil i.e. bulk soil may see reduced influence of rhizo-deposits, which have been shown to follow a gradient, and so impact less on micro-organism diversity (Marilley et al. 1998, Kandeler et al. 2002,

Gomes et al. 2003). Within this study the total number of Glomermycota taxa sequenced, across both sites, was 12 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 of ~300 base pairs, whilst adequate for Ascomycota and Basidiomycota species differentiation (Cole et al. 2014), has been criticised as not adequately differentiating Glomermycota species (Stockinger, Kruger & Schussler 2010, Brown et al. 2013, Kemler et al. 2013). The release of 400 base pair chips may help to rectify this, and allow far greater resolution of soil fungi, coupled with primers targeting Glomermycota specifically (Kruger et al. 2009).

There was an increase in the abundance of the one MF taxon, *Rhizophagus* sp. (phylum Glomermycota; order Glomerales), a constituent of the Biagro® Grass (Plantworks Ltd. oral comm.). However, it was not possible within this dataset to discern if the increased abundance of the fungi was of native or applied origin. Interestingly though, there was a negative correlation between soil phosphate and the abundance of the fungi within the BG-treated grass plots, while control plot abundance remained largely unaffected by increasing P. Several reports have shown how non-native MF species may not be acclimatised to various edaphic factors (Lambert, Cole & Baker 1980, Enkhtuya, Rdlová & Vosátka 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.

The application of some BIs within this study were found to significantly alter fungal community assemblages. The application rate was also found to be a significant factor at the Gadlas site, in which Biagro® Grass at ten times application rates was found to significantly affect the relative abundance of fungal taxa. Intrestingly grass roots of plots from the ten times application rate exhibited increased mycorrhizal colonisation for BG-treated grass (Chapter 5). Increased abundances of some taxa between treated and un-treated plots such as *Tricladium* sp. (phylum Ascomycota; order Helotiales) at GA and *Veronaea* (phylum Ascomycota; order Chaetothyriales) at MG. The latter location also saw reduced abundances of some taxa post BI application, *Pyrenochaeta* sp. (phylum Ascomycota; order Pleosporales) and *Coprinopsis* sp. (phylum Basidiomycota; order Agaricales). *Tricladium* sp. are part of the Helotiales order which are predominantly saprobes of soil humus and decaying matter (Wang et al. 2006).

Overall, however, there were no significant changes in the fungal diversity or equitability measures of BI application at either rate (recommended and ten times recommended). The application rates of the BIs applied to the treatment plots were very low, as little as 125 g ha^{-1} (Biagro® S) including carrier material, and it is unlikely that such small quantities of spores and root fragments would impact on the established fungal communities. The BIs impacting on initial plant root development possibly (Chapter 3) as such further work examining the roots of plants post BI application may yield more distinct differences (Dumbrell et al. 2011).

7.6.1. NGS technology

Ion Torrent™ has both strengths and weaknesses (Shokralla et al. 2012), the high scale sequencing of environmental samples, and multiple sample comparisons make it a very cost effective way of examining changes in soil fungal community assemblages. In the past the cost of generating sequence data was a limiting factor in the use of NGS technologies for the analysis of microbial communities. As sequencing costs continue to fall, the use of NGS platforms will no doubt increase, as the potential for these technologies to further improve our knowledge of the complex interactions of micro-organisms is impressive. NGS platform can help remove problems associated with traditional morphological examinations (Carew et al. 2013) or difficulties in growing many species in axenic culture (Brown et al. 2013) for example.

The sequencing technique used allowed very detailed sequencing down to the genus level, which allowed for a more detailed comparison between treatments and sites, 77% fungi identified to family, 65% to genus. The weaknesses stem from the potential PCR bias during amplification, although all multiplexing technologies will be subject to this. However, comparison of the LSU primers used for this study with reference sequences from GenBank have failed to identify any primer mismatches that would cause bias against certain taxa. Sample preparation is also very time consuming but it is difficult to avoid these since the PowerSoil® method contains several steps which are necessary to remove humic and other compounds which are inhibitory to PCR. Future developments will reduce biases and enhance and simplify NGS protocols. Future developments both in the hardware and software will further refine data, especially as reference barcodes are obtained for a wider range of fungi. Further investigations utilising NGS technology of long term fertilised plots, for example, would be of interest to explore the sensitivities of individual taxa to perturbations in their environment from both abiotic and biotic factors. The technology could also be used in parallel

with other microbial analysis such as ergosterol assay, which would quantify fungal biomass (Clemmensen et al. 2013; Wallander et al. 2013). Ergosterol is an important sterol in the membrane of most fungi and can be used as an estimate of living fungal biomass, the membrane is quickly lysed and its components decompose upon hyphal death (Newell, 2001).

Conclusion

The study was able to show the potential of NGS technology, Ion Torrent™, for examining changes in micro-organisms within the field. The study found that nitrogen application and seasonality affected fungal abundances significantly, whilst the application of bio-inoculants at the recommended application rates did alter the abundance of some fungal taxa, did not alter the overall fungal community. Phosphate availability appears to have had a neglible effect on fungal communities and would require further study.

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CHAPTER 8: GENERAL DISCUSSION

8.1. General discussion

This thesis had several specific aims: to explore the effectiveness of some specific commercial bio-inoculants (BIs) on grass growth within both the laboratory and the field, to ascertain the contribution of phosphorus (P) with respect to any BI mediated yield gains and to establish any changes in soil microbial composition after BI application. The implications of the findings of the research are discussed.

Over the past decade, interest in BIs has increased greatly, a response born of many factors from a shift towards reducing the reliance of agriculture on finite raw-material resources used in inorganic fertiliser manufacture, to a concerted effort to reduce the environmental impact of agriculture (EU COM, 2011). Better awareness of the roles of micro-organisms within the soil and plant root rhizosphere have seen huge increases in both the development and use of commercial BI products, but it remains very unclear how effective they are and by what specific mechanisms. Chapter 2 evaluated the results of efficacy tests of some commercial BIs while also trying to clarify the nomenclature associated with BIs, in relation to their mode of action for plant-growth enhancement. The lack of peer-reviewed research within the literature examining commercial BIs efficacy within field locations was highlighted.

The legal wranglings of regulation and licencing of commercial products begins with semantics; for example defining "what" such products are - bio-fertilisers or bio-inoculants. Although many market bio-fertiliser products as anything which contains living organisms (Vessey 2003); as proposed in Chapter 2 a bio-fertiliser be more rigorously defined as a product which may contain micro- / macro-plant nutrients, and / or specific organic components which, directly or indirectly, stimulate microbial activity to increase the mobilisation of plant-beneficial nutrients. A bio-inoculant, as a product containing individual strains, or consortia, of known microbes that have potential plant growth-promoting benefits. The two are not mutually exclusive and could feature within the same product as a bio-resource. Several of the commercial products utilised within this study contained both a bio-fertiliser and bio-inoculant fraction as per the definitions laid out in Chapter 2.

The non-living fraction of some of the bio-resource products were shown to exert plant benefits (Chapter 3-4), and while the products could technically be deemed a bio resource, hereafter BI refers to commercial products whose main marketable selling point is the fact they contain micro-organisms.

The five research chapters' aims were largely satisfied, in that the efficacy trials of the BI products were found to increase grass yields both in the laboratory (Chapter 3) and to a smaller extent in the field (Chapter 5-6). P mobilisation of recalcitrant P, both inorganic and organic, was demonstrated in the laboratory (Chapter 4), whilst the interactive effects of various P-fertilisers and BIs were successfully assessed both in the laboratory and the field (Chapter 4 & 6). Large microbial DNA sequence datasets generated from field soils of the trial sites gave an insight into the effects of various management practices, including BI application, on soil fungi (Chapter 7).

The research highlighted the need to exercise caution when extrapolating laboratory-based results to field-scale applications. For example, many products are marketed as increasing plant yields with an emphasis on improving plant-available phosphorus. Within this research we were able to demonstrate, both within the laboratory and within the field, the mobilisation of P. Within the controlled conditions of the laboratory all BIs tested performed well, mobilising P successfully from both inorganic and, to a lesser extent, organic sources (Chapter 4). BI treatments increased several plant growth parameters considerably, such as shoot and root growth (Chapter 3). Within the laboratory it was found that P-mediated yield gains varied between BIs, dependent on what type of P fertiliser was applied (Chapter 4). However, in the field P-mediated yield gains from BIs were less pronounced (Chapter 6) and interactive effects between BIs and phosphate fertiliser were not found at all.

The lower effectiveness of BIs on grass yield and P uptake under field conditions may be due to many factors impacting on efficacy trials, ranging from weather conditions, to pedalogical variations between sites. The soils of both field trial sites contained a diverse community of fungi (Chapter 7), and abiotic factors were found to impact significantly on those communities, from soil type variation and seasonality to the application of inorganic nitrogen fertiliser, more so than the application of BIs or soil P status. While yields of the grasses were slightly increased with BI application the overall effect on bulk soil fungal communities was negligible. The re-establishment of native populations of fungi after the disruption of tillage, for example, possibly began to nullify the initial effects of BI application as non-treated grasses "caught-up" (Chapter 5). This suggests that the treatments provide some gains to plants at establishment, through increased rooting for example (Chapter 3), or increased nutrient acquisition (Chapter 4), but across a season the effects of BIs begin to fade as the more established native populations re-assert their dominance. Obviously from a commercial perspective this would be regarded as a positive, in terms of repeated applications.

Although results exhibited high variability, some BIs did show signs of prolonged plant benefit after initial application by increasing yields across several cuts: for example both SSI and PN-treated grass yielded higher than non-treated controls (Chapter 6). When one considers the application rate of the BIs, especially the fungal based products which were as low as 0.125 kg ha^{-1}, these results reflect quite positively on the potential viability of the living component of the BIs.

A major selling point of many commercial BIs is their ability to increase the availability and plant acquisition of legacy P. Many products are aimed at soils of initially low P and / or high P-fixation and soils with a relatively high level of plant-available P may therefore not see any added benefits from BI application (Chapter 5). Within this study, it was found that BI-treated grasses did not exhibit any improved yields with increasing plant-available P, both within the laboratory (Chapter 4) and the field (Chapter 6). Indeed, increasing soil P was found to have a negative effect on the abundance of one taxon of BI-treated grass plots, *Rhizophagus* sp. (Chapter 7). At low levels of available P, the abundance of this widely utilised fungi within BI manufacture was significantly greater than that of control plots. Furthermore, the abundance was found to be negatively correlated with increasing plant-available soil P, unlike native populations which remained unchanged. This highlights the variability in community response of added BIs to site conditions, even within the same genus of MF, due to their lack of prior acclimatisation to specific soil conditions (Khan et al. 2009). The correlation however is not necessarily an indication of cause and effect, and phosphate level represented only 8% of the variability, the abundance of the fungi affected by other factors as well as phosphate availability.

The significant increase in abundance of *Rhizophagus* sp. is further evidence in support of the viability of the living component of the BIs, in spite of low application rates, to proliferate when soil conditions are favourable. The result also highlights the potential of the NGS DNA sequencing technology. Although the Ion Torrent™ was able to discern differences down to genus level between soils, it was not possible, within this study, to differentiate between native and applied fungi within the treatment plots, as such claims regards persistence of applied fungi are not robust.

There are mixed reports within the literature of P-availability effects on soil fungi (Beauregard et al. 2010, Wang et al. 2015). Within this study, P-availability (P-index, Chapter 7) had a non-significant effect on fungal community assemblages. Levels of soil-P may have more impact

on rhizospheric than that of bulk soil fungal communities (Wang et al. 2015). The lack of response warrants further investigation to establish if fungal community within soil is P-pool sensitive, for example plant-available, organic and total P.

The research poses some interesting questions regarding the future direction of BIs. A changing legal framework of registration for BIs likely to be introduced, potentially requiring information such as species specific labelling, provenance and proof of efficacy etc. (Malussa 2014), which could impact on the manufacturing process.

For example, one of the inoculants utilised within this study was stated as containing one taxa of MF, *Rhizophagus irregularis* (formerly *Glomus intraradices*); but as well as the MF other fungi were detected, *Mortierella* sp. (Chapter 4 & 7), whilst within the consortia BI, *Trichoderma* sp. were present (Glenside, oral comm. & Chapter 7). Whilst both are known P-mobilising fungi, the former has also been identified as a potential bio-control agent (Edgington et al. 2014) and latterly as a myco-fungicide (Kaewchai, Soytong & Hyde 2009), as such both could be classified as a bio-pesticide (Malussa 2014), subject to different regulatory standards and registration (EU Regulation (EC) 1107/2009).

Trap culture methods employed by many manufacturers are thought to contain background populations of micro-organisms of ~10^3 organisms per gram of BI. High sequence DNA sequencing platforms, such as the Ion Torrent^{TM}, are getting cheaper and could also allow manufacturers a rapid method for maintaining consistency and maintaining quality control within BI manufacture. Many manufacturers also employ a monoxenic culture method, which involves root organ cultures. This method produces pure spores of a particular MF, however as previously mentioned, there exists huge variation between MF cultured in various soil environments, this could equally apply to those cultured in soil-less environments. This is an area that should be explored further.

The development of new standards e.g. under the ISO Technical committee 276 on biotechnology could pave the way for a more robust standard of products in which the viability of spores are guaranteed; for example in India, MF products must be able to provide 80 infection points per gram of inoculum (Malusa, Vassilev 2014). However as seen in Chapter 4, higher mycorrhizal infection is not necessarily correlated with yield increases. A new set of regulatory standards to include efficacy studies specifying crop used, soil management and period of BI application has been proposed (Malusa, Vassilev 2014).

There exists huge variation between BI effectiveness and fungal soil communities between soils. A potential future development could also be BI manufacture based on soil samples from the field of interest. Plant-beneficial organisms could be identified, isolated and cultured and a bespoke BI manufactured to the specific soil environment.

The results suggest soils with adequate levels of plant-available P may not see much benefit to warrant the application of BIs, or at least at the application rates recommended. While the field trials, may not have strengthened the case for the use of BIs, with respect to P, the study was able to highlight the positive impact mycorrhizae have on other aspects of plant growth, such as increased N acquisition and restricted uptake on elements such as aluminium for example. Further work is required to improve effectiveness and consistency of BIs across variable abiotic and biotic soil environments. The results of this study therefore question the idea of a universal BI. The variability exhibited between products does little to re-enforce consumer confidence, and while manufacturers currently create products containing a suite of micro-organisms to encourage use across varying abiotic and biotic environments, it is quite feasible that the functional redundancy of current BIs will become redundant in the face of new regulations.

8.2. Further work

8.2.1. Products

The work highlighted the complexity of trying to investigate BI effectiveness. Within this work and that of many others, commercial BIs have been shown to differ greatly in their ability to perform within various growing media and varying application rates (Corkidi et al. 2004, Rowe, Brown & Claassen 2007, Tarbell, Koske 2007).

Future work should focus on enhancing product consistency through the development of a robust repeatable testing procedure which could combine field soil variability and the consistency of laboratory conditions, allowing a cost effective way to assess BIs. One area of assessment could be the development of a P mobilisation quantification system in which the total P mobilisation (inorganic and organic) potential of a BI is quantified within optimal conditions.

As was seen within this study (Chapter 3), and already practised in some countries, the use of inert carrier media, such as zeolite, attapulgite and vermiculite have significant effects on soil, plants and fungi. Further investigations of the impact of inert carrier media on soil bacterial

and fungal communities and the potential synergistic effects with fungal and bacterial BI constituents is warranted. A multi-discipline approach involving material science offers an exciting area of research in the development of new more effective carrier products.

Application rates was found to be more of a significant factor below ground (Chapter 5 and 7) than above ground. Examining the relationship between fugal species, propagule numbers and persistence within the soil would contribute to the development of more effective fungal based BI products, which could improve above ground responses. There was some evidence to suggest variations between native and applied taxa of the same genus (Chapter 7) for example. Individual strains of MF within the soil could be monitored after application, using technologies like NGS and egresterol which could both identify and quantify respectively the effectiveness of applied fungi.

As was seen within this study (Chapter 5-6), control plots exhibited extensive native mycorrhizal activity, and due to the ubiquity of the MF-plant symbioses globally, and the shared evolutionary history between fungi and plants, it is reasonable to assume that a "non-infected" control plant, as used in laboratory studies, could be regarded as not a control, but a treatment (Smith, Smith 2012); and for a true and representative efficacy test one should be using plants with a standard level of MF infection. This would require an agreed standard of mycorrhizal infectivity, which would need to encompass a host of agriculturally important crops within a wide variety of abiotic and biotic factors. The complexity of creating such a standard would be immense.

8.2.2. Application and use

Within this study there was much variability both between and within treatments, due to space limitations, often replicate numbers were low (ranging from 3-9), increased numbers of replicates would be desirable in any future field trials to further reduce variability and increase confidence.

Refinement of the manufacturing process, especially trap culture method, may reduce contamination of unwanted organisms (Chapter 4), indeed depending on the robustness of proposed regulatory standards may be a requirement. Integration of NGS sequencing technology into BI design and manufacture could reduce contamination within soil based growth systems. Soilless systems, such as the monoxenic culture method, should also be investigated. Comparative studies exploring the variations in fungal effectiveness between manufacturing processes.

Management conditions within this study had a significant effect on soil fungi (Chapter 7), there was a mixed response of fungal taxa abundance to nutrient availability for example. Fungal communities and individual taxa within the soils of this study appear to be less influenced by phosphate (Chapter 7) than other nutrients such as nitrogen (Chapter 7). A key area of future research should be to explore the magnitude of the response of soil fungi to management perturbations and potential interactions between nutrients such as nitrogen and phosphorus. Furthermore the lack of a P-effect on soil fungi within the field requires further investigation. An examination of rhizospheric and bulk soil fungal community changes in response to P-pool availability, total and palnt-avaialbe for example, may help to explain the lack of a P-effect; and would further our understanding of P-cycling within soil.

There are numerous other edaphic factors which could also be investigated, such as water availability and micro-nutrient availability. For example, the relationship between mycorrhizal colonisation and plant biomass aluminium, iron (Chapter 5) and other micro-nutrient nutrients (Chapter 6) and the mechanisms involved and implications to plant yields warrants further investigation.

BIs are marketed as a component of sustainable agriculture and are regarded as an important component within the EU strategy of reducing the environmental impact of the sector; as such the attitudes of the agricultural sector towards BIs could provide valuable insight into perceptions of BIs, and any issues regards limited / no adoption of BI products could be assessed and addressed. Recent studies have found large variations in attitudes of farmers, for example, to climate change and the impact that their sector has and adoption of mitigation strategies (Hyland et al. 2015). Overall there is much work to do to both within science and industry in the development and manufacture of bio-inoculants to improve standards and confidence in the sector.

8.3. References

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APPENDIX

9.1. TXRF - elemental analysis

BIs and growth media were subjected to total reflection X-ray fluorescence (TXRF), which offers a precise determination of major and trace elements within samples. Samples were ground to pass a 63 µm sieve, ~ 20 mg was then suspended in 1 ml of 1% Triton-X solution. An internal standard, Selenium, was added (10 µl of 1000 µg ml^{-1}) giving an internal standard mass of 10 µg. After homogenisation an aliquot of 10 µl was transferred to a siliconized quartz glass sample carrier and dried on a heating plate. Quartz glass discs, 30 mm diameter and a thickness of 3 mm ± 0.1 mm were applied as TXRF sample carriers. Sample carriers were previously siliconized by 10 µl of a silicon solution in isopropanol to avoid spreading of the samples across the surface of the carriers. Measurements were performed using a benchtop S2 PICOFOX™ TXRF-spectrometer (Bruker AXS Microanalysis GmbH, Germany). Equipped with a metal-ceramic X-ray tube with a Mo-anode and air cooling, a planar multilayer monochromator (Ni / C), and XFlash® Si drift detector with an area of 30 mm^2 and < 150 eV resolution for the Mn K_α-line. Measurements were conducted at 50 kV operating voltage and 750 µA current. The measurement time was 1000 s per sample. The treatment of the X-ray spectra and analysis of the separate fluorescence peak overlaps were performed using SPECTRA 6.1 software.

9.2. Soil analysis

9.2.1. Organic matter (OM)

Organic matter (OM) content was measured by placing oven-dried samples into muffle furnace (400 °C). OM measured as the difference in weight between oven dried and ashed sample.

9.2.2. pH

pH was determined on a 1:5 (soil : deionised water) suspension. Measurement made using a glass electrode/reference electrode system using a pH meter standardised against known buffer solutions.

9.2.3. Electrical conductivity (EC)

Electrical conductivity (EC) is a measure of the ionic activity of a solution, and gives an indication of nutrient concentrations within a soil sample. EC measured using the standard potentiometric method on an EC meter.

9.2.4. Water holding capacity (WHC)

Water holding capacity (WHC) was measured as the amount of water held within an oven dried (105 °C) soil sample after 24 hours. Normal field WHC is ~ 60-80%, corresponding to the optimal biological activity for WHC.

9.3. Nutrient Solutions

Nutrient composition of Hoagland's solution. *20% of what is usually added 1M KCl added to maintain ionic balance.

Component	**Stock Solution ($g\ l^{-1}$)**	**Hoagland's Solution ($ml\ l^{-1}$)**
Macronutrients		
2M KNO_3	202	2.5
1M $Ca(NO_3)_2 \cdot 4H_2O$	118	2.5
Iron (Sprint 138 iron chelate)	15	1.5
2M $MgSO_4 \cdot 7H_2O$	493	1
1M NH4NO3	80	1
1M KH_2PO_4	136	0.10*
1M KCl	74.6	0.4
Micronutrients		
H_3BO_3	2.86	11
$MnCl_2 \cdot 4H_2O$	1.81	1
$ZnSO_4 \cdot 7H_2O$	0.22	1
$CuSO_4 \cdot 5H_2O$	0.051	1
$Na_2MoO_4 \cdot 2H_2O$	0.12	1

9.4. Chapter 3 – Article I

9.4.1. Total length of root classes

Total root length (cm) and individual root class lengths (cm). Values in parenthesis are ± 1 standard deviation. Values with an asterisk indicate significant difference as compared to the control (Anova, LSD post hoc, $p < 0.05$).

		Total length (cm) of individual root class widths (mm)					
BI	**Total (cm)**	**> 0.01 - < 0.1**	**> 0.1 - < 0.2**	**> 0.2 - < 0.3**	**> 0.3 - < 0.4**	**> 0.4 - < 0.5**	**> 0.5**
Control	641 (± 16)	335 (± 13)	138 (± 6)	77 (± 8)	28 (± 2)	23* (± 7)	38 (± 9)
Biagro® PhosN	2341* (± 352)	1099* (± 146)	516* (±103)	312* (± 58)	111* (± 16)	85* (± 10)	211* (± 46)
Biagro® MP	1886* (± 721)	942* (± 351)	428* (± 186)	235* (± 85)	77* (± 25)	58* (± 19)	141* (± 70)
Biagro® Grass	1937* (± 573)	1056* (± 289)	433* (± 134)	220* (± 69)	70* (± 26)	50* (± 22)	104* (± 45)
SSI	2230* (± 825)	1116* (± 383)	507* (± 219)	293* (± 120)	92* (± 38)	70* (± 20)	145* (± 63)
Biagro® S	1695* (± 275)	884* (± 172)	393* (± 71)	200* (± 26)	65* (± 9)	48* (± 5)	102* (± 16)

9.4.2. Sterilisation on root length classes

Individual root class lengths (cm) of sterilised and non-sterilised bio-inoculant treatments. Values in parenthesis are ± 1 standard deviation. Values with an asterisk are found to be significantly affected by sterilisation (Anova, LSD post hoc, $p < 0.05$).

			Total length (cm) of individual root class widths (mm)					
Treatment	**BI**	**Total (cm)**	**> 0.01 - < 0.1**	**> 0.1 - < 0.2**	**> 0.2 - < 0.3**	**> 0.3 - < 0.4**	**> 0.4 - < 0.5**	**> 0.5**
Sterilised	*PN*	298 (± 259)	165 (± 142)	51 (± 43)	44 (± 32)	11 (± 10.4)	7 (± 8)	18 (± 23)
	BMP	974 (± 501)	488 (± 261)	221 (± 99)	119 (± 68)	50 (±27)	36 (± 20)	59 (± 40)
	BG	1202 (± 179)	551 (± 82)	236 (± 46)	167 (± 24)	63 (± 8)	48 (± 9)	134 (± 15)
	SSI	1854 (± 171)	927 (± 129)	394 (± 60)	227 (± 22)	79 (± 5)	59 (± 6)	164 (± 34)
	BS	2500 (± 406)	1334 (± 258)	470 (± 75)	287 (± 41)	104 (± 15)	81 (± 14)	217 (± 16)
Non-sterilised	*PN*	2341* (± 353)	1099* (± 146)	516* (±103)	312* (± 58)	111* (± 16)	85* (± 10)	211* (± 46)
	BMP	1886* (± 721)	942* (± 351)	428* (± 186)	235* (± 85)	77 (± 25)	58 (± 19)	141* (± 70)
	BG	1937 (± 573)	1056* (± 289)	433* (± 134)	220 (± 69)	70 (± 26)	50 (± 22)	104 (± 45)
	SSI	2230 (± 825)	1116 (± 383)	507 (± 219)	293 (± 120)	92 (± 38)	70 (± 20)	145 (± 63)
	BS	1695* (± 275)	884* (± 172)	393 (± 71)	200 (± 26)	65* (± 9)	48* (± 5)	102* (± 16)

9.4.3. Statistical analysis – Sterilisation on root classes

Statistical multivariate analysis of the total root length and six root class width orders and the interactive effect between sterilisation and BI treatment. *p* considered significant at < 0.05 level, F values in parenthesis.

		Individual root class widths					
Factor	**Total**	**< 0.1**	**< 0.2**	**< 0.3**	**< 0.4**	**< 0.5**	**> 0.5**
Sterilisation	< 0.001 (14.9)	< 0.001 (15.2)	< 0.001 (18.0)	< 0.001 (14.2)	< 0.05 (9.0)	< 0.05 (10.7)	0.140 (2.3)
*Sterilisation*BI*	< 0.001 (7.4)	< 0.001 (7.4)	< 0.05 (4.2)	< 0.001 (6.7)	< 0.001 (9.8)	< 0.001 (12.9)	< 0.001 (12.8)

9.4.4. Sterilisation effects

Sterilised and non-sterilised treatments. Superscript letters within each measured variable denote a significant difference (Anova, LSD post hoc, $p < 0.05$). Asterisk data was transformed to maintain homogeneity of variance.

	Treatment	**Mean**	*Std. Deviation*
Shoot DMY (g)	*Control*	.052	.010
	Sterilised	.107	.063
	Non-sterilised	.125	.044
Root DMY* (g)	*Control*	.018[a]	.004
	Sterilised	.055[b]	.038
	Non-sterilised	.073[c]	.028
% P in Shoot	*Control*	.202	.029
	Sterilised	.129	.040
	Non-sterilised	.152	.044
Total P (g)	*Control*	.00012[a]	.000008
	Sterilised	.00015[a]	.000071
	Non-sterilised	.00021[b]	.000071
Total root length (cm)	*Control*	641.18[a]	16.38
	Sterilised	1365.78[b]	829.14
	Non-sterilised	2011.01[c]	606.26
< 0.1* (cm)	*Control*	335.07[a]	12.94
	Sterilised	692.97[b]	445.13
	Non-sterilised	1018.76[c]	285.23
< 0.2 (cm)	*Control*	138.36[a]	6.40
	Sterilised	274.52[a]	161.75
	Non-sterilised	457.36[b]	152.33
< 0.3 (cm)	*Control*	77.24[a]	8.38
	Sterilised	168.65[b]	93.84
	Non-sterilised	250.57[c]	85.61

	Treatment	Mean	*Std. Deviation*
< 0.4 (cm)	*Control*	28.28[a]	2.49
	Sterilised	61.38[b]	34.54
	Non-sterilised	81.48[c]	28.84
< 0.5 (cm)	*Control*	23.04[a]	6.79
	Sterilised	46.04[b]	27.33
	Non-sterilised	61.14[c]	21.05
> 0.5 (cm)	*Control*	37.55	8.89
	Sterilised	118.32	77.61
	Non-sterilised	136.42	61.82
Total Lateral length (0 – 0.2) (cm)	*Control*	473[a]	12
	Sterilised	967[b]	603
	Non-sterilised	1475[c]	416
Total seminal length (0.2 – 0.5) (cm)	*Control*	166[a]	7
	Sterilised	394[b]	229
	Non-sterilised	538[c]	189
Lateral : Seminal	*Control*	2.85[ab]	0.13
	Sterilised	2.55[b]	0.53
	Non-sterilised	2.86[a]	0.57
Surface area (cm^2)	*Control*	33.66[a]	1.91
	Sterilised	84.64[b]	50.87
	Non-sterilised	112.39[c]	40.22
Lateral surface area (LSA) (cm^2)	*Control*	13.23[a]	0.37
	Sterilised	26.05[a]	15.88
	Non-sterilised	41.36[b]	12.49

	Treatment	Mean	*Std. Deviation*
Seminal surface area (SSA) (cm^2)	*Control*	20.43[a]	2.18
	Sterilised	58.59[b]	35.81
	Non-sterilised	71.03[c]	29.32
LSA : SSA	*Control*	2.85	0.13
	Sterilised	2.55	0.53
	Non-sterilised	2.90	0.57
Root volume (cm^3)	*Control*	0.212[a]	.019
	Sterilised	0.635[b]	.391
	Non-sterilised	0.766[c]	.330
Density (g. cm^3)	*Control*	0.083	.015
	Sterilised	0.083	.024
	Non-sterilised	0.094	.014
Root length density (cm. cm^{-3} Soil)	*Control*	2.62[a]	.067
	Sterilised	5.57[b]	3.38
	Non-sterilised	8.24[c]	2.40
Specific root length (SRL)*	*Control*	37.69	10.16
	Sterilised	34.34	21.69
	Non-sterilised	30.18	7.39
PER (g DMY mg^{-1} P)	*Control*	0.502[a]	.078
	Sterilised	0.843[c]	.250
	Non-sterilised	0.704[b]	.180

9.5. Chapter 4 – Article II

9.5.1. Grass Harvesting

Harvesting of grass units six weeks after planting. Grass was cut by hand to ~ 1 cm above the soil.

a. Six week growth

b. After harvest

9.5.2. Triple super phosphate (TSP) and Rock phosphate (RP)

Inorganic P fertilisers applied to growth units.

a. Triple super phosphate (TSP) (46% P_2O_5)

b. Rock phosphate (RP) – Highland Slag (15% P_2O_5)

9.6. Chapter 5

9.6.1. Field trial plot layout

Individual plot layout, 4 m^2 (red box). All plant and soil samples taken from within the 1 m^2 (yellow box). Photo taken prior to first yield cut (July 2013).

9.6.2. Second cut – yield analysis

Second cut, mycorrhizal frequency of colonisation (F%), intensity of colonisation (M%), dry matter yield (DMY) (t ha^{-1}), % phosphorus (P) of the forage, total forage P (kg ha^{-1}), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P) . Values in parenthesis are ± 1 standard deviation.

Application rate	**Treatment**	**DMY** (t ha^{-1})	**F %**	**M%**	**% P**	**Total forage P** (kg)	**PER** (g DM mg^{-1} P)
Recommended	*Control*	3.93 (± 0.50)	88.9 (± 7.7)	28.6 (± 19.1)	0.31 (± 0.06)	12.21 (± 3.55)	0.33 (± 0.07)
	BG	4.63 (± 0.29)	88.9 (± 11.7)	40.9 (± 1.2)	0.30 (± 0.10)	13.72 (± 3.95)	0.36 (± 0.12)
	BS	4.16 (± 0.39)	92.2 (± 8.4)	33.6 (± 13.3)	0.38 (± 0.19)	15.80 (± 7.74)	0.31 (± 0.16)
	BMP	4.21 (± 1.07)	86.9 (± 11.7)	27.3 (± 12.2)	0.32 (± 0.03)	13.67 (± 4.47)	0.31 (± 0.03)
×10	*Control*	3.63 (± 0.69)	97.8 (± 3.9)	46.9 (± 11.6)	0.53 (± 0.10)	19.16 (± 3.88)	0.19 (± 0.04)
	BG	3.81 (± 0.34)	88.4 (± 10.8)	37.0 (± 21.4)	0.41 (± 0.12)	15.29 (± 3.60)	0.26 (± 0.10)
	BS	3.88 (± 0.61)	93.3 (± 6.7)	45.8 (± 9.6)	0.38 (± 0.08)	14.72 (± 4.15)	0.27 (± 0.06)
	BMP	4.06 (± 0.66)	91.2 (± 8.9)	39.6 (± 14.0)	0.33 (± 0.10)	14.21 (± 8.21)	0.32 (± 0.09)

9.6.3. Three cuts – yield analysis

Dry matter yield (DMY) (t ha^{-1}) of three individual cuts and the mean total, inoculated with three BIs, at two application rates, recommended and ×10. The % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P) (means of all three cuts, n = 9). Frequency of mycorrhizal colonisation (F%) and intensity (M%) (1st and 2nd cut mean total); the establishment cut was not examined for MF activity, as it was cut < six weeks after seeding and MF require up to ~12 weeks to colonise roots (INVAM, personal comm.). Values in parenthesis are ± 1 standard deviation. Different superscript letters represent significant differences between BIs within both cut and application rate (Anova, LSD post hoc, $p < 0.05$).

		Cut (DMY) (t ha^{-1})								
Application rate	**Treatment**	**Establishment**	**1st**	**2nd**	**Total**	**F%**	**M%**	**% P**	**Total forage P (kg)**	**PER (g DM mg^{-1} P)**
Recommended	*Control*	0.43 (± 0.21)	3.84[a] (± 0.35)	3.93 (± 0.50)	8.19 (± 0.94)	71.1 (± 22.1)	19.5 (± 15.9)	0.31 (± 0.04)	25.85 (± 4.33)	0.32 (± 0.03)
	BG	0.52 (± 0.43)	5.00[b] (± 0.70)	4.63 (± 0.29)	10.15 (± 0.85)	72.7 (± 23.7)	24.4 (± 15.5)	0.31 (± 0.09)	28.39 (± 6.01)	0.37 (± 0.11)
	BS	0.59 (± 0.20)	3.66[a] (± 0.35)	4.16 (± 0.39)	8.41 (± 0.78)	69.3 (± 26.1)	21.2 (± 16.3)	0.34 (± 0.10)	29.20 (± 5.79)	0.30 (± 0.07)
	BMP	0.44 (± 0.21)	3.72[a] (± 0.27)	4.21 (± 1.07)	8.37 (± 1.01)	62.3 (± 29.0)	16.3 (± 14.6)	0.31 (± 0.03)	26.17 (± 5.42)	0.32 (± 0.03)
×10	*Control*	0.51 (± 0.15)	3.37 (± 0.73)	3.63 (± 0.69)	7.51 (± 1.15)	60.1 (± 41.5)	25.8 (± 24.4)	0.40 (± 0.13)	31.24 (± 3.39)	0.24 (± 0.05)
	BG	0.49 (± 0.11)	3.88 (± 0.17)	3.81 (± 0.34)	8.18 (± 0.39)	68.7 (± 23.1)	23.4 (± 20.3)	0.35 (± 0.09)	28.79 (± 4.86)	0.29 (± 0.06)
	BS	0.39 (± 0.04)	3.53 (± 0.39)	3.88 (± 0.61)	7.80 (± 1.02)	60.7 (± 36.1)	25.3 (± 23.3)	0.34 (± 0.06)	27.33 (± 4.47)	0.29 (± 0.04)
	BMP	0.32 (± 0.23)	3.66 (± 0.42)	4.06 (± 0.66)	8.04 (± 0.96)	58.5 (± 41.3)	22.5 (± 21.1)	0.33 (± 0.06)	27.11 (± 8.07)	0.31 (± 0.05)

9.6.4. Macro- / micro-element forage analysis

Macro (%) and micro (mg kg^{-1}) elemental analysis of first cut, totals are the mean (n = 6) of BIs for both application rates (recommended and × 10). Values in parenthesis are ± 1 standard deviation.

	Macro-element (%)					**Micro-element (mg kg^{-1})**								
BI	**Mg**	**Ca**	**Na**	**K**	**S**	**Mn**	**Cu**	**Zn**	**Se**	**Co**	**I**	**Fe**	**Al**	**Mo**
Control	0.14 (± 0.03)	0.43 (± 0.11)	0.05 (± 0.02)	2.96 (± 0.37)	0.23 (± 0.03)	106.1 (± 38.6)	6.67 (± 1.75)	26.17 (± 5.05)	0.04 (± 0.02)	0.07 (± 0.02)	0.32 (± 0.08)	100.2 (± 31.5)	33.8 (± 15.1)	1.02 (± 0.38)
Biagro® Grass	0.13 (± 0.02)	0.44 (± 0.07)	0.04 (± 0.01)	2.62 (± 0.46)	0.21 (± 0.03)	97.5 (± 28.2)	6.03 (± 0.44)	29.02 (± 4.35)	0.04 (± 0.02)	0.05 (± 0.02)	0.24 (± 0.04)	72.0 (± 6.9)	17.8 (± 2.1)	0.82 (± 0.26)
Biagro® S	0.13 (± 0.02)	0.41 (± 0.09)	0.06 (± 0.03)	2.95 (± 0.26)	0.23 (± 0.04)	109.2 (± 40.1)	5.62 (± 0.88)	25.08 (± 3.69)	0.04 (± 0.01)	0.06 (± 0.02)	0.25 (± 0.08)	79.7 (± 13.2)	24.5 (± 6.0)	0.98 (± 0.25)
Biagro® MP	0.13 (± 0.02)	0.43 (± 0.05)	0.05 (± 0.03)	2.75 (± 0.30)	0.22 (± 0.02)	111.2 (± 29.0)	5.92 (± 0.39)	25.82 (± 3.11)	0.04 (± 0.01)	0.06 (± 0.02)	0.27 (± 0.10)	87.7 (± 35.1)	28.8 (± 16.2)	0.97 (± 0.21)

9.6.5. Nutrient analysis of individual of treatment strips

Soil analysis of the entire site, as presented in Table 5.1 (section 5.4.1), measured NO_3 and NH_4 at 23 mg kg^{-1} and 12 mg kg^{-1} respectively. Soil analysis of each treatment strip (Recommended and ten times recommended rates), of the three main plant beneficial nutrients, N, P and K as measured by NRM (Berkshire, UK) found that the recommended treatment strip was almost two fold higher in NH_4-N concentration. Figures based on pooled samples of soil cores but were not replicated, as such no statistical tests performed.

Table 5.3. Soil characteristics of individual application rate treatment strips, showing ammonium (NH_4), nitrate (NO_3), total and plant-available phosphorus (P) and total potassium (K) (NRM Ltd., Berkshire, UK). Values represent pooled samples of 12 cores per treatment strip

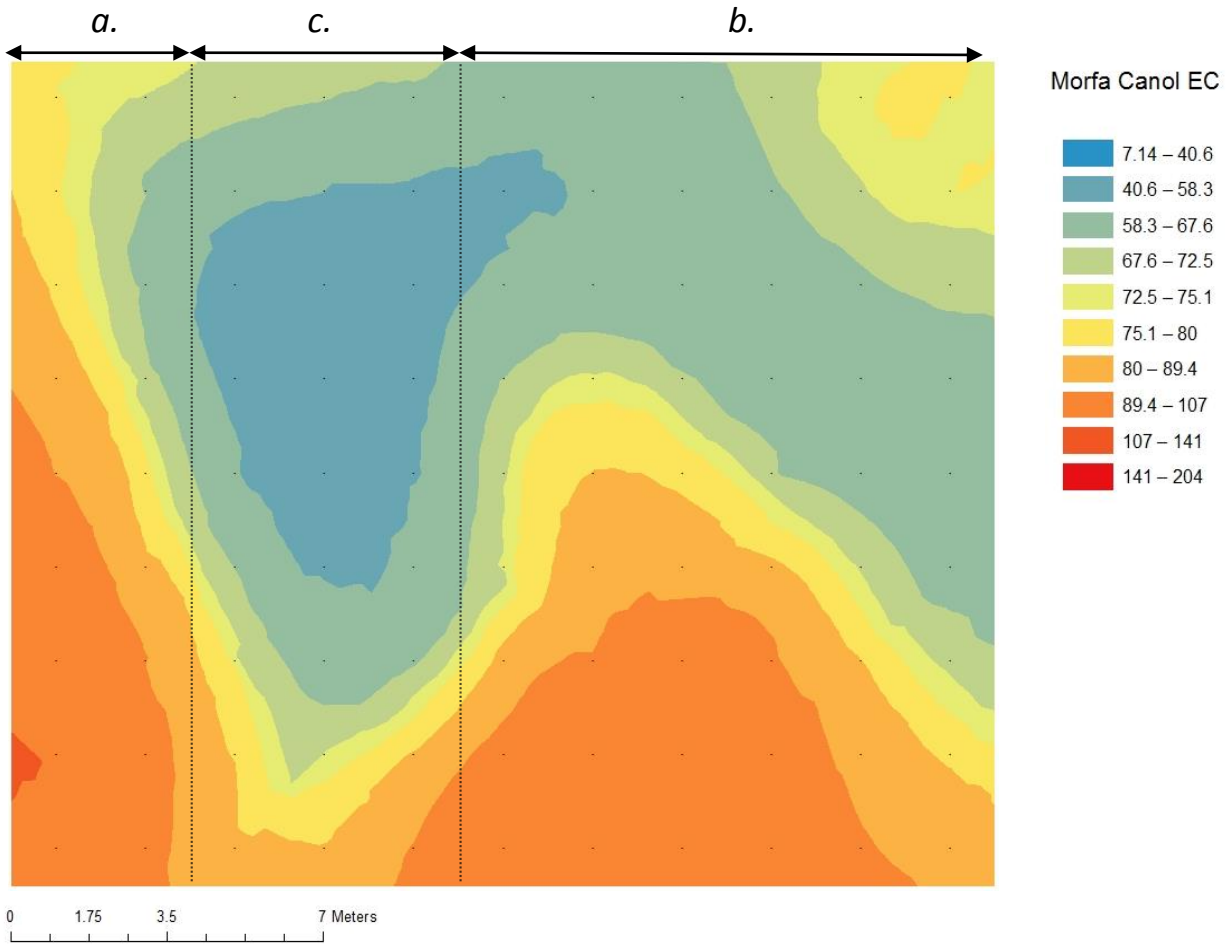
	N[x]			**P**		**K**	
Treatment	**NO_3-N (mg kg^{-1})**	**NH_4-N (mg kg^{-1})**	**Available* (kg ha^{-1})**	**Total (mg kg^{-1})**	**PO_4-P (mg kg^{-1})**	**Total (mg kg^{-1})**	**Available (mg kg^{-1})**
Recommended	10	35	178	1208	22	857	159
×10	15	19	134	1275	21	973	167

*30 cm profile depth

9.7. Chapter 6 – Article IV

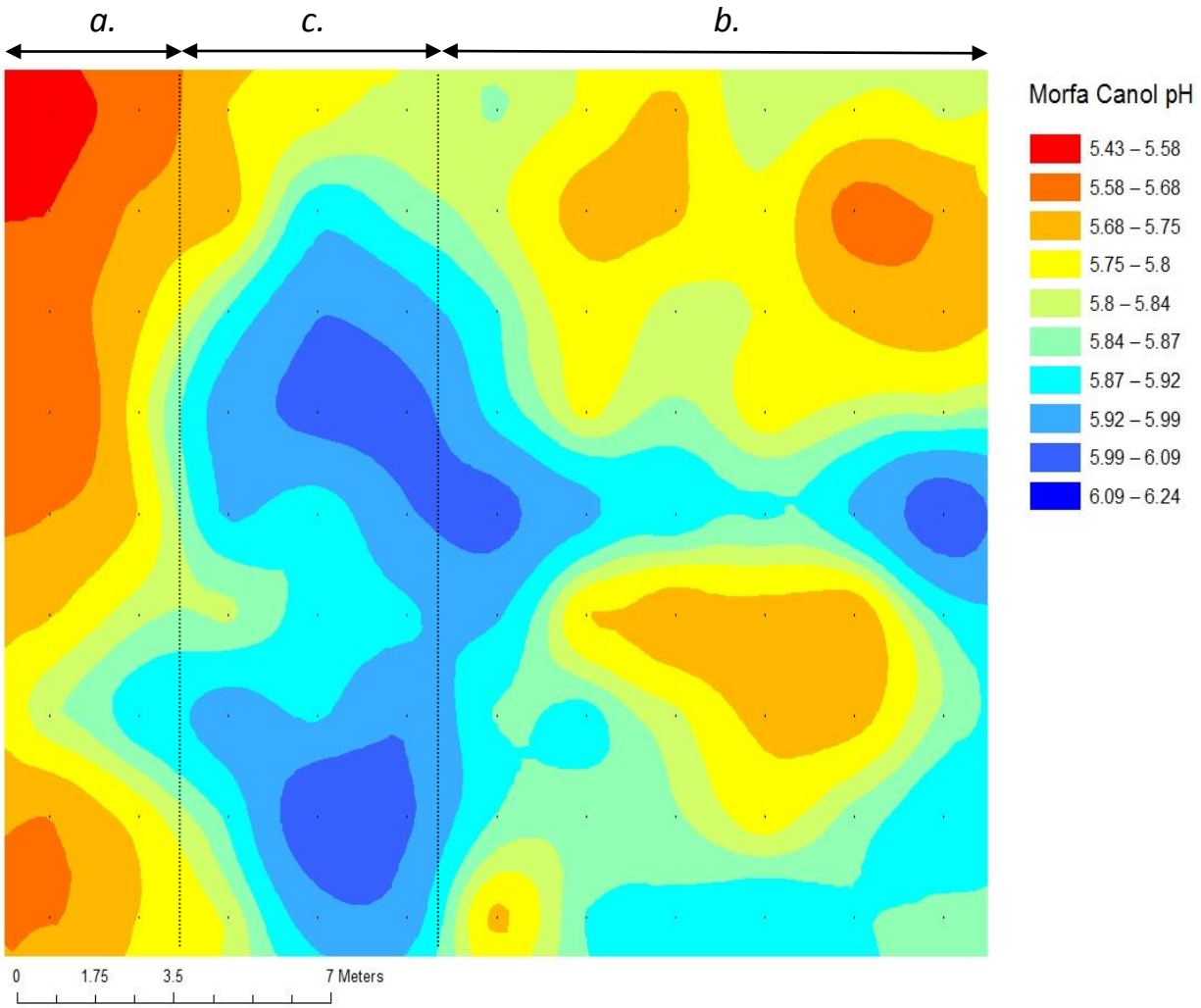
9.7.1. GIS - Soil electrical conductivity (EC)

Soil EC across the Morfa Ganol experimental site. GIS krigging of individual data points (*n* = 99). *a*) P-index trial and *b)* P-fertiliser trial, section marked *c*. was not utilised.



9.7.2. Soil pH

Soil pH across the Morfa Ganol experimental site. GIS krigging of individual data points (*n* = 99). *a*) P-index trial and *b)* P-fertiliser trial, section marked *c*. was not utilised.



9.7.3. P-gradient – Individual cuts

Individual cuts of P-gradient trial, dry matter yield (DMY) (t ha^{-1}), % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P) of Biagro® Grass (BG) treated grass and control plots (n = 9), across two cuts. Values in parenthesis are ± 1 standard deviation.

Cut	Treatment	DMY (kg ha^{-1})	% P	Total forage P (kg)	PER (g DM mg^{-1} P)
1	BG	6307 (± 1172)	0.20 (± 0.03)	12.4 (± 2.6)	0.52 (± 0.07)
	Control	6240 (± 787)	0.19 (± 0.02)	12.0 (± 2.0)	0.52 (± 0.05)
2	BG	960 (± 180)	0.22 (± 0.03)	2.1 (± 0.6)	0.47 (± 0.07)
	Control	1134 (± 182)	0.20 (± 0.03)	2.3 (± 0.5)	0.51 (± 0.07)

9.7.4. P-source – Second cut

Second cut, dry matter yield (DMY) (t ha^{-1}), % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P). Different superscript letters represent significant differences between BI-treated grass of each P-treatment (n = 3) (Anova, LSD post hoc, $p < 0.05$), different capital letters represent significant differences between P treatments mean totals (Anova, LSD post hoc, $p < 0.05$). Values in parenthesis are ± 1 standard deviation.

P treatment	BI	DMY (kg ha^{-1})	% P	Total forage P (kg)	PER (g DM mg^{-1} P)
TSP	*Control*	3918 (± 873)	0.32^{a} (± 0.09)	13.0 (± 6.1)	0.33 (± 0.09)
	BG	4506 (± 360)	0.23^{ab} (± 0.04)	10.6 (± 1.2)	0.43 (± 0.06)
	SSI	5516 (± 270)	0.19^{b} (± 0.02)	10.5 (± 0.4)	0.52 (± 0.04)
	PN	5359 (± 1920)	0.24^{ab} (± 0.09)	13.1 (± 6.1)	0.44 (± 0.14)
	Mean	**4824 (± 1143)**	**0.25 (± 0.08)**	**11.8 (± 3.9)**	**0.43A (± 0.11)**
RP	*Control*	5127 (± 222)	0.17 (± 0.02)	8.9 (± 0.7)	0.58 (± 0.05)
	BG	4481 (± 219)	0.22 (± 0.07)	10.0 (± 2.7)	0.47 (± 0.14)
	SSI	5389 (± 960)	0.16 (± 0.01)	8.3 (± 1.3)	0.64 (± 0.01)
	PN	5646 (± 1746)	0.17 (± 0.03)	10.0 (± 4.1)	0.58 (± 0.09)
	Mean	**5161 (± 972)**	**0.18 (± 0.04)**	**9.3 (± 2.3)**	**0.57B (± 0.10)**
No P	*Control*	4290 (± 557)	0.18 (± 0.01)	7.7 (± 1.3)	0.56 (± 0.03)
	BG	6078 (± 1362)	0.22 (± 0.10)	14.0 (± 9.2)	0.51 (± 0.18)
	SSI	5483 (± 627)	0.16 (± 0.02)	9.0 (± 0.9)	0.62 (± 0.05)
	PN	5556 (± 1514)	0.19 (± 0.02)	10.8 (± 4.0)	0.54 (± 0.08)
	Mean	**5394 (± 1187)**	**0.19 (± 0.05)**	**10.4 (± 5.0)**	**0.56B (± 0.10)**

9.7.5. P-source – Third cut

Third cut, dry matter yield (DMY) (t ha^{-1}), % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P). Different superscript letters represent significant differences between BI-treated grass of each P-treatment (n = 3) (Anova, LSD post hoc, $p < 0.05$). Values in parenthesis are ± 1 standard deviation.

P treatment	BI	DMY (kg ha^{-1})	% P	Total forage P (kg)	PER (g DM mg^{-1} P)
TSP	*Control*	1239 (± 109)	0.20 (± 0.07)	2.4 (± 1.1)	0.57 (± 0.20)
	BG	1435 (± 337)	0.16 (± 0.03)	2.4 (±1.0)	0.64 (± 0.12)
	SSI	1628 (± 380)	0.16 (± 0.06)	2.6 (± 1.0)	0.70 (± 0.28)
	PN	1594 (± 157)	0.19 (±0.03)	3.0 (± 0.7)	0.55 (± 0.09)
	Mean	**1474 (± 282)**	**0.18 (± 0.05)**	**2.6 (± 0.9)**	**0.61 (± 0.17)**
RP	*Control*	1259^{a} (± 239)	0.15 (± 0.04)	2.0 (± 0.8)	0.68 (± 0.15)
	BG	1238^{a} (± 188)	0.16 (± 0.01)	2.0 (± 0.3)	0.61 (± 0.02)
	SSI	1712^{b} (± 27)	0.14 (± 0.02)	2.5 (± 0.4)	0.71 (± 0.10)
	PN	1821^{b} (± 97)	0.16 (± 0.02)	3.0 (± 0.2)	0.63 (± 0.07)
	Mean	**1508 (± 306)**	**0.16 (± 0.02)**	**2.3 (± 0.6)**	**0.66 (± 0.09)**
No P	*Control*	1461^{ab} (± 194)	0.18 (± 0.04)	2.6 (± 0.8)	0.58 (± 0.12)
	BG	1301^{b} (± 197)	0.14 (± 0.03)	1.8 (± 0.6)	0.73 (± 0.13)
	SSI	1378^{b} (± 142)	0.16 (± 0.02)	2.3 (± 0.5)	0.62 (± 0.08)
	PN	1765^{a} (± 140)	0.15 (± 0.02)	2.6 (± 0.4)	0.69 (± 0.09)
	Mean	**1476 (± 234)**	**0.16 (± 0.03)**	**2.4 (± 0.7)**	**0.62 (± 0.10)**

9.7.6. P-source – Total (second and third cut)

Total (second and third cut) dry matter yield (DMY) (kg ha^{-1}) of MG*b*, % phosphorus (P) of the forage, total forage P (kg), and phosphorus efficiency ratio (PER) (g DM mg^{-1} P). Values in parenthesis are ± 1 standard deviation. Different superscript letters represent significant differences between BI-treated grass of each P-treatment (n = 3) (Anova, LSD post hoc, p < 0.05). Values in parenthesis are ± 1 standard deviation

P treatment	**BI**	**DMY (kg ha^{-1})**	**% P**	**Total forage P (kg)**	**PER (g DM mg^{-1} P)**
TSP	Control	5157 (± 949)	0.29 (± 0.09)	15.4 (± 7.1)	0.37 (± 0.11)
	BG	5941 (± 121)	0.22 (± 0.03)	12.9 (± 1.7)	0.47 (± 0.07)
	SSI	7143 (± 219)	0.18 (± 0.01)	13.1 (± 1.3)	0.55 (± 0.04)
	PN	6953 (± 1833)	0.23 (± 0.07)	16.1 (± 6.7)	0.46 (± 0.13)
RP	Control	6386 (± 361)	0.17 (± 0.02)	10.9 (± 1.5)	0.59 (± 0.06)
	BG	5719 (± 117)	0.21 (± 0.05)	12.0 (± 2.7)	0.49 (± 0.12)
	SSI	7107 (± 977)	0.15 (± 0.003)	10.8 (± 1.7)	0.66 (± 0.02)
	PN	7468 (± 1751)	0.17 (± 0.02)	12.9 (± 4.3)	0.59 (± 0.09)
No P	Control	5751 (± 750)	0.18 (± 0.01)	10.3 (± 2.1)	0.56 (± 0.05)
	BG	7379 (± 1558)	0.21 (± 0.09)	15.9 (± 9.8)	0.54 (± 0.18)
	SSI	6924 (± 874)	0.16 (± 0.01)	11.2 (± 1.0)	0.61 (± 0.03)
	PN	7428 (± 1665)	0.18 (± 0.02)	13.4 (± 4.4)	0.57 (± 0.08)

9.7.7. Nitrogen and Phosphorus ratio (N : P)

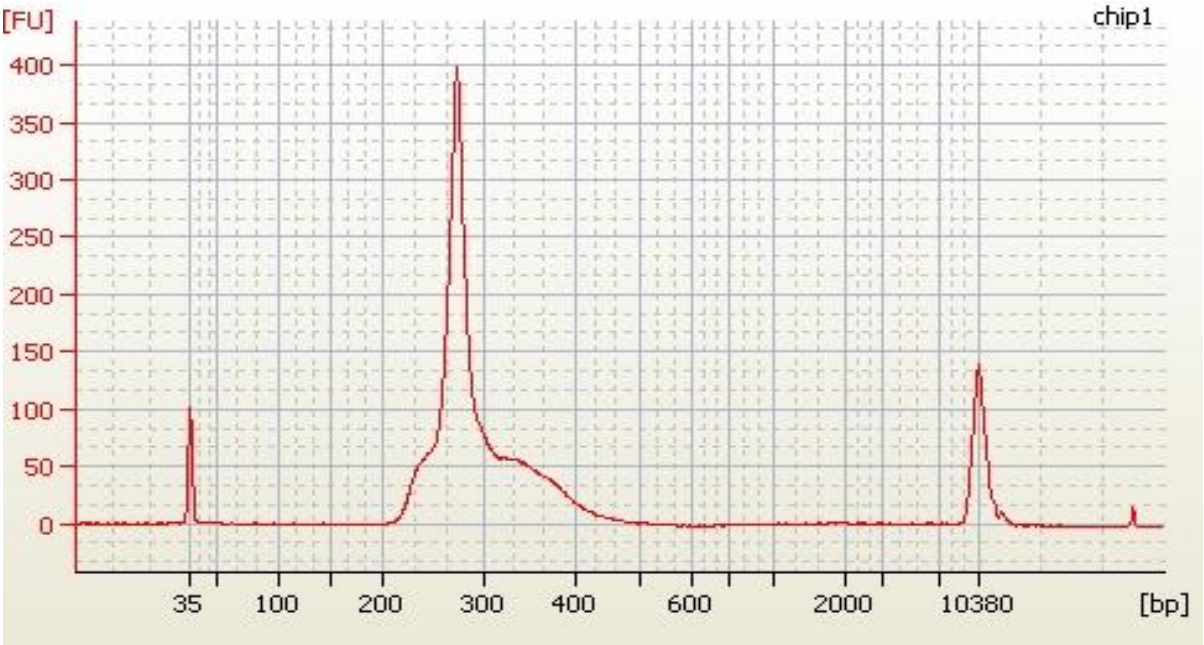
N : P ratios of 2nd and 3rd cuts (after P-treatment application). Different superscript letters represent significant differences between P treatments (Anova, LSD post hoc, $p < 0.05$).Values in parenthesis are ± 1 standard deviation.

		Cut	
P-treatment	**Bio-inoculant**	**2**	**3**
TSP	*Control*	8.86 (± 3.60)	8.08 (± 4.12)
	Biagro® Grass	9.77 (± 2.13)	8.15 (± 1.81)
	SSI	9.87 (± 3.11)	7.93 (± 3.33)
	Biagro® PhosN	9.37 (± 2.03)	7.00 (± 1.35)
	Mean	**9.47[a] (± 2.42)**	**7.79 (± 2.51)**
RP	*Control*	12.31 (± 0.60)	8.47 (± 2.02)
	Biagro® Grass	12.48 (± 3.59)	7.70 (± 0.23)
	SSI	12.38 (± 2.45)	8.25 (± 1.67)
	Biagro® PhosN	13.26 (± 2.65)	6.86 (± 0.31)
	Mean	**12.61[b] (± 2.22)**	**7.82 (± 1.30)**
0 P	*Control*	14.24 (± 0.64)	7.14 (± 1.18)
	Biagro® Grass	10.26 (± 1.70)	7.48 (± 0.52)
	SSI	15.44 (± 1.76)	7.06 (± 0.62)
	Biagro® PhosN	13.02 (± 2.11)	8.27 (± 1.20)
	Mean	**13.24[b] (± 2.45)**	**7.49 (± 0.94)**

9.8. Chapter 7 – Article V

9.8.1. Bio-analyser trace

Output from the bio-analyser trace ensuring that DNA is at ~300 base pairs, the two smaller peaks are instrument calibrations. Trace shows that there are no short fragments that may adversely affect sequencing.



9.8.2. Ion Torrent phylum abundances – All samples

All sample data of both sites. Totals, individual totals for each site and standard deviations. Gadlas (GA) and Morfa Ganol (MG).

Abundance (%)	**Total**	*Std. dev*	**GA** (*n* = 83)	*Std. dev*	**MG** (*n* = 85)	*Std. dev*
Fungi identified to family	76.67	*10.29*	75.19	*9.58*	78.12	*10.79*
Fungi identified to genus	65.43	*11.30*	63.56	*10.77*	67.26	*11.57*
Ascomycota	51.86	*15.26*	49.09	*13.93*	54.56	*16.08*
Basidiomycota	32.69	*15.78*	26.85	*13.57*	38.39	*15.77*
Blastocladiomycota	0.12	*0.81*	0.23	*1.15*	0.02	*0.04*
Chytridiomycota	2.03	*1.97*	3.30	*2.09*	0.80	*0.59*
Fungi incertae sedis	7.70	*7.37*	12.51	*7.73*	3.00	*2.14*
Glomeromycota	1.09	*1.07*	1.42	*1.32*	0.77	*0.60*
Not identified	4.50	*3.25*	6.59	*3.24*	2.45	*1.48*
Fungi Total	23962	*18828*	17400	*17111*	30370	*18298*
Non Fungi Total	4524	*5729*	5177	*7599*	3886	*2837*
Taxa Count	306	*132*	265	*132*	346	*120*
OTU Count	372	*171*	320	*172*	423	*155*
Shannon Index (*H*)	3.76	*0.52*	3.80	*0.55*	3.71	*0.48*
Equitability (E_H)	0.65	*0.08*	0.68	*0.07*	0.62	*0.08*

9.8.3. Taxa abundance at both sites – Gadlas & Morfa Ganol

Top 200 OTU relative abundance (%) mean totals sequenced across all sample of both experimental sites and individual site totals (%) (Gadlas (GA) (*n* = 83) and Morfa Ganol (MG) (*n* = 85)).

Phylum	Class	Order	Family	Genus	Count	Total	GA	MG
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	168	8.86	4.19	13.41
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	168	6.19	8.26	4.16
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	166	4.11	7.88	0.42
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	168	3.07	1.66	4.44
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	168	2.95	4.11	1.82
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 55	130	2.78	3.25	2.32
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	167	2.58	1.95	3.18
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Pyrenochaeta	168	2.35	3.43	1.29
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus_ter	168	2.29	1.29	3.26
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Gamsiella	158	2.28	3.41	1.17
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	165	1.93	1.15	2.69
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	168	1.90	2.60	1.21
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 17	167	1.80	1.82	1.77
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	146	1.58	0.06	3.05
Ascomycota	Leotiomycetes	Helotiales	X	OTU 16	135	1.47	0.99	1.94
Basidiomycota	Agaricomycetes	X	X	OTU 14225	62	1.41	0.01	2.78
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OTU 9	79	1.40	2.68	0.15
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Conocybe	94	1.37	0.11	2.60

Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OTU 5866	26	0.98	0.00	1.93
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Panaeolus	163	0.96	0.71	1.20
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium complex	162	0.88	1.27	0.50
Ascomycota	X	X	X	OTU 5	156	0.87	1.35	0.41
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Graddonia	121	0.85	1.05	0.64
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Massariosphaeria	167	0.78	1.17	0.39
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Orbicula	18	0.77	0.00	1.53
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Piriformospora	144	0.75	1.10	0.40
X	X	X	X	OTU 11	160	0.74	1.41	0.08
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lachnum	132	0.72	0.34	1.09
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Waitea	78	0.68	0.69	0.66
Basidiomycota	Tremellomycetes	Tremellales	X	OTU 99	121	0.65	1.27	0.05
Basidiomycota	Microbotryomycetes	Microbotryomycetes incertae sedis	Microbotryomycetes incertae sedis	Kriegeria	165	0.65	0.41	0.89
Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	Ascobolus	166	0.64	0.80	0.48
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 69	101	0.63	0.87	0.40
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Lipomyces_tetrasporus	168	0.56	0.58	0.55
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Alphamycetaceae	Betamyces	167	0.55	0.91	0.20
X	X	X	X	OTU 14	97	0.53	1.01	0.05
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	OTU 19	85	0.53	1.04	0.03
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Mollisia	160	0.53	0.72	0.34
Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	uncultured_Thelephoraceae	159	0.47	0.29	0.65
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Saccharomyces_kudriavzevii	45	0.45	0.04	0.85
Ascomycota	Leotiomycetes	X	X	OTU 97	111	0.45	0.23	0.66

Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	OTU 2097	40	0.44	0.71	0.18
Basidiomycota	X	X	X	OTU 155	132	0.44	0.19	0.69
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Uthatobasidium	47	0.43	0.81	0.05
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Rickenella	144	0.42	0.54	0.31
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 6398	22	0.41	0.00	0.81
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacina	150	0.40	0.43	0.38
Fungi incertae sedis	Mucoromycotina	Endogonales	Endogonaceae	Endogone	148	0.40	0.26	0.53
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	OTU 7968	4	0.39	0.79	0.00
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Tetracladium	166	0.38	0.43	0.34
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	OTU 3337	50	0.38	0.00	0.75
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 6045	22	0.37	0.00	0.74
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Nolanea	151	0.36	0.12	0.59
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 931	85	0.34	0.00	0.67
X	X	X	X	OTU 102	161	0.34	0.30	0.37
Ascomycota	Dothideomycetes	Pleosporales	Montagnulaceae	OTU 241	104	0.34	0.01	0.66
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeosphaeria	44	0.31	0.08	0.53
Chytridiomycota	Chytridiomycetes	X	X	OTU 89	142	0.31	0.57	0.06
Ascomycota	Leotiomycetes	Helotiales	X	OTU 52	77	0.30	0.52	0.09
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	Mrakia	137	0.30	0.57	0.03
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	Trechispora	141	0.28	0.07	0.48
Ascomycota	Laboulbeniomycetes	Pyxidiophorales	Pyxidiophoraceae	Pyxidiophora	158	0.27	0.36	0.19
Basidiomycota	Agaricostilbomycetes	Agaricostilbales	Chionosphaeraceae	Kurtzmanomyces	119	0.27	0.05	0.48
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	157	0.27	0.40	0.14
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Endophyte	58	0.27	0.12	0.42
X	X	X	X	OTU 1783	59	0.26	0.53	0.00

Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Ceratobasidium	73	0.26	0.05	0.46
Basidiomycota	Agaricomycetes	X	X	OTU 161	65	0.24	0.45	0.04
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	OTU 38	95	0.24	0.41	0.07
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Flagelloscypha	56	0.24	0.28	0.20
Basidiomycota	Agaricomycetes	X	X	OTU 506	107	0.23	0.14	0.32
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	OTU 3375	73	0.23	0.00	0.45
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OTU 3238	17	0.22	0.25	0.20
Chytridiomycota	Chytridiomycetes	X	X	OTU 78	102	0.22	0.25	0.20
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 14953	14	0.22	0.43	0.01
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 3382	41	0.22	0.00	0.43
Basidiomycota	Agaricomycetes	X	X	OTU 213	47	0.21	0.43	0.00
Basidiomycota	Atractiellomycetes	Atractiellales	Hoehnelomycetaceae	Atractiella	125	0.21	0.25	0.17
Basidiomycota	Agaricomycetes	X	X	OTU 5089	30	0.21	0.00	0.41
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	114	0.20	0.29	0.12
Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	Sporobolomyces	138	0.20	0.29	0.12
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Monilinia	83	0.20	0.22	0.18
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Cudoniella	123	0.19	0.11	0.28
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 14203	47	0.18	0.00	0.36
Basidiomycota	Microbotryomycetes	X	X	OTU 748	54	0.18	0.36	0.01
Ascomycota	Leotiomycetes	Helotiales	X	OTU 3442	74	0.18	0.01	0.35
Basidiomycota	Agaricomycetes	Agaricales	Marasmiaceae	Crinipellis	1	0.18	0.00	0.35
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Melanotus	16	0.18	0.00	0.35
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OTU 12438	2	0.17	0.35	0.00
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Pseudorobillarda	69	0.17	0.00	0.33
Basidiomycota	Agaricomycetes	Geastrales	Sphaerobolaceae	Sphaerobolus	39	0.16	0.00	0.32

Glomeromycota	Glomeromycetes	Archaeosporales	Archaeosporaceae	Archaeospora	138	0.16	0.24	0.09
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Calyptella	26	0.16	0.03	0.29
X	X	X	X	OTU 289	100	0.16	0.01	0.31
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 2282	99	0.16	0.02	0.28
Basidiomycota	Agaricomycetes	Agaricales	Lycoperdaceae	Lycoperdon	25	0.15	0.00	0.30
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Camarosporium	44	0.15	0.00	0.30
Ascomycota	Leotiomycetes	Leotiomycetes incertae sedis	Myxotrichaceae	OTU 3668	70	0.15	0.00	0.29
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 164	53	0.15	0.29	0.01
Ascomycota	Leotiomycetes	Helotiales	X	OTU 83	81	0.15	0.21	0.09
Ascomycota	Dothideomycetes	Capnodiales	Capnodiales incertae sedis	Pseudoramichloridium	87	0.15	0.00	0.29
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus	97	0.14	0.12	0.17
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida_sake_(T)	92	0.14	0.02	0.25
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	Columnosphaeria	75	0.14	0.27	0.01
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	OTU 2607	25	0.13	0.01	0.26
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiales incertae sedis	Leucosporidium	73	0.13	0.26	0.01
Ascomycota	Leotiomycetes	Helotiales	X	OTU 9354	8	0.13	0.00	0.25
Basidiomycota	X	X	X	OTU 6068	48	0.13	0.25	0.01
Ascomycota	Pezizomycetes	Pezizales	Ascodesmidaceae	Ascodesmis	150	0.13	0.09	0.17
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Neofabraea	57	0.13	0.25	0.01
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Pseudombrophila	144	0.12	0.06	0.19
Blastocladiomycota	Blastocladiomycetes	Blastocladiales	Catenariaceae	Catenomyces	91	0.12	0.23	0.02
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 6403	25	0.12	0.07	0.17
Basidiomycota	Agaricomycetes	X	X	OTU 2120	8	0.12	0.24	0.01
Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	OTU 2718	18	0.12	0.24	0.00

Ascomycota	Eurotiomycetes	Verrucariales	Verrucariaceae	OTU 2056	16	0.12	0.01	0.23
X	X	X	X	OTU 1747	131	0.12	0.14	0.10
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 2612	24	0.12	0.22	0.02
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 120	110	0.12	0.08	0.15
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_2	116	0.12	0.20	0.03
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_3	143	0.11	0.09	0.13
Ascomycota	Leotiomycetes	X	X	OTU 119	59	0.11	0.21	0.01
Ascomycota	X	X	X	OTU 534	8	0.11	0.22	0.00
Basidiomycota	Agaricomycetes	Thelephorales	Bankeraceae	OTU 16188	4	0.11	0.00	0.21
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	Operculomyces	132	0.10	0.15	0.06
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Mucor	55	0.10	0.13	0.08
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 533	107	0.10	0.12	0.08
Basidiomycota	Classiculomycetes	Classiculales	Classiculaceae	Classicula	145	0.10	0.10	0.10
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Spirosphaera	125	0.10	0.04	0.15
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Davidiella	30	0.10	0.13	0.07
Ascomycota	X	X	X	OTU 2113	21	0.10	0.20	0.00
Basidiomycota	Agaricomycetes	X	X	OTU 2163	90	0.09	0.01	0.18
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Mycosphaerella	4	0.09	0.19	0.00
Ascomycota	Leotiomycetes	Helotiales	X	OTU 22402	2	0.09	0.00	0.19
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	OTU 3067	16	0.09	0.02	0.17
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Eupenicillium	125	0.09	0.03	0.16
Basidiomycota	Agaricomycetes	Auriculariales	Exidiaceae	Endoperplexa	49	0.09	0.18	0.00
Ascomycota	Leotiomycetes	X	X	OTU 3563	24	0.09	0.18	0.00
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Lamprospora	109	0.09	0.04	0.14
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Mycena	82	0.09	0.01	0.16

Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Tubaria	12	0.09	0.01	0.16
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 14493	44	0.09	0.04	0.13
Ascomycota	Leotiomycetes	Helotiales	X	OTU 17583	2	0.09	0.00	0.17
Ascomycota	Leotiomycetes	Helotiales	X	OTU 9163	12	0.09	0.09	0.08
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 14053	10	0.08	0.04	0.13
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Thanatephorus	59	0.08	0.00	0.16
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 71	68	0.08	0.16	0.01
Ascomycota	Dothideomycetes	Pleosporales	Lophiostomataceae	OTU 569	121	0.08	0.09	0.07
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Boeremia	39	0.08	0.08	0.08
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Torrendiella	94	0.08	0.08	0.08
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	OTU 12516	3	0.08	0.16	0.00
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Issatchenkia_orientalis_ ATCC_6258_(T)	10	0.08	0.15	0.01
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	OTU 165	94	0.07	0.01	0.14
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 5636	5	0.07	0.00	0.15
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 13735	5	0.07	0.15	0.00
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	86	0.07	0.15	0.00
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Paurocotylis	14	0.07	0.14	0.01
X	X	X	X	OTU 593	78	0.07	0.09	0.05
Basidiomycota	Exobasidiomycetes	Georgefischeriales	Tilletiariaceae	Tilletiaria	126	0.07	0.12	0.02
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 2257	6	0.07	0.14	0.00
Basidiomycota	Agaricomycetes	Cantharellales	Clavulinaceae	OTU 10269	23	0.07	0.01	0.13
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus	40	0.07	0.11	0.03
Ascomycota	Dothideomycetes	Microthyriales	Microthyriaceae	Microthyrium	96	0.07	0.14	0.01
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	Melanomma	97	0.07	0.08	0.06

Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVX1	113	0.07	0.04	0.10
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 310	120	0.07	0.04	0.09
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	OTU 3408	12	0.07	0.00	0.13
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinellus	27	0.07	0.13	0.01
Ascomycota	Ascomycota incertae sedis	Ascomycota incertae sedis	Ascomycota incertae sedis	Troposporella	70	0.07	0.13	0.00
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 1065	95	0.07	0.00	0.13
X	X	X	X	OTU 40	51	0.07	0.13	0.00
Ascomycota	X	X	X	OTU 613	80	0.07	0.05	0.08
Basidiomycota	Agaricomycetes	X	X	OTU 2477	73	0.06	0.05	0.08
Chytridiomycota	Chytridiomycetes	Cladochytriales	Nowakowskiellaceae	Nowakowskiella	140	0.06	0.10	0.03
Basidiomycota	Agaricomycetes	X	X	OTU 5524	16	0.06	0.00	0.13
Basidiomycota	Pucciniomycetes	Platygloeales	Eocronartiaceae	Eocronartium	91	0.06	0.10	0.03
Basidiomycota	Agaricostilbomycetes	Spiculogloeales	Spiculogloeales incertae sedis	Mycogloea	130	0.06	0.10	0.03
X	X	X	X	OTU 108	113	0.06	0.11	0.01
Basidiomycota	Agaricomycetes	Boletales	Stephanosporaceae	Stephanospora	42	0.06	0.11	0.02
Basidiomycota	Agaricomycetes	Agaricomycetes incertae sedis	Agaricomycetes incertae sedis	Tricellulortus	65	0.06	0.10	0.02
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_4	114	0.06	0.09	0.03
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Pilidium	28	0.06	0.12	0.00
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 4033	4	0.06	0.00	0.11
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	69	0.06	0.02	0.10
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Byssonectria	78	0.06	0.07	0.04
X	X	X	X	OTU 146	134	0.06	0.06	0.06
Ascomycota	X	X	X	OTU 25	26	0.06	0.11	0.00
Ascomycota	Eurotiomycetes	Onygenales	Arachnomycetaceae	Arachnomyces	93	0.06	0.02	0.09

Basidiomycota	X	X	X	OTU 8696	12	0.06	0.04	0.07
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Clathrosporium	18	0.06	0.11	0.00
X	X	X	X	OTU 2190	4	0.06	0.11	0.00
Ascomycota	Leotiomycetes	Helotiales	X	OTU 2224	17	0.06	0.11	0.01
X	X	X	X	OTU 126	27	0.06	0.11	0.01
Ascomycota	Dothideomycetes	Pleosporales	Lentitheciaceae	Keissleriella	57	0.06	0.03	0.09
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	OTU 14717	5	0.06	0.09	0.02
X	X	X	X	OTU 58	58	0.06	0.11	0.00
Basidiomycota	Agaricomycetes	X	X	OTU 5913	7	0.06	0.00	0.11
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVAR	94	0.05	0.06	0.05
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Clitopilus	27	0.05	0.04	0.07
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Octospora	97	0.05	0.06	0.05

9.8.4. Phylum abundance at different sampling date – Morfa Ganol

Morfa Ganol (MG) site samples taken in July (n = 5) and September (n = 3) 2013. Showing the top 19 fungal sequences and the mean, median (med), maximum (max) and minimum (min) relative abundance (%) of the Ion Torrent output. Totals for each phylum and % of sequences identified to family and genus. Asterisk indicates significant difference between sampling dates (Anova, $p < 0.05$).

					July				Sept			
Phylum	**Class**	**Order**	**Family**	**Genus**	*mean*	*med.*	*max.*	*min.*	*mean*	*med.*	*max.*	*min.*
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea*	9.89	9.63	12.40	7.39	16.09	16.34	20.48	11.45
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium*	2.81	2.98	4.33	1.55	7.86	7.17	10.99	5.43
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	3.48	3.06	6.62	1.25	5.38	4.24	10.05	1.87
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 6045	6.63	5.49	19.29	0.00	0.00	0.00	0.00	0.00
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Conocybe	5.92	3.32	17.87	0.22	1.08	0.02	3.21	0.01
Basidiomycota	Agaricomycetes	Agaricales	Marasmiaceae	Crinipellis	6.02	0.00	30.08	0.00	0.00	0.00	0.00	0.00
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus_ter	3.81	3.71	5.45	2.07	2.82	2.66	3.66	2.16
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 17	2.97	3.21	5.08	0.79	3.72	3.52	4.26	3.37
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	3.30	2.20	6.40	2.05	1.68	1.05	3.20	0.77
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	2.77	1.93	5.72	0.78	2.51	2.67	2.93	1.95
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	2.88	3.16	4.61	1.39	2.10	2.25	2.97	1.08
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	2.78	2.12	4.67	0.83	2.26	2.39	2.89	1.50
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Gamsiella	2.25	1.57	6.02	0.20	1.78	2.07	2.15	1.13
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Tubaria	2.39	0.00	11.93	0.00	0.00	0.00	0.00	0.00
Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Uncultured _Thelephoraceae	1.69	1.16	3.42	0.81	0.74	0.64	0.98	0.59
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Panaeolus	1.83	0.74	6.51	0.31	0.33	0.38	0.46	0.15
Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	Ascobolus	0.72	0.43	2.18	0.11	1.97	1.82	3.06	1.02
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 5636	1.84	0.00	7.04	0.00	0.00	0.00	0.00	0.00
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 55	0.33	0.24	0.81	0.00	2.18	1.82	4.18	0.55
		Totals			*mean*	*med.*	*max.*	*min.*	*mean*	*med.*	*max.*	*min.*
		% Fungi id. to family			82.1	82.4	92.5	71.2	81.8	85.2	85.8	74.2
		% Fungi id. to genus			72.5	70.6	88.6	64.9	69.7	67.5	75.2	66.3
		Ascomycota*			42.7	45.9	52.8	29.1	66.8	73.3	78.1	49.1
		Basidiomycota*			51.1	46.2	61.4	41.9	17.6	14.4	25.7	12.5
		Blastocladiomycota			0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
		Chytridiomycota			0.6	0.5	1.1	0.3	2.0	1.2	3.9	0.9
		Fungi incertae sedis			3.1	2.4	7.2	0.6	5.7	4.9	9.4	2.9
		Glomeromycota*			0.2	0.2	0.3	0.1	1.6	1.1	3.0	0.6
		Not identified			2.2	2.3	3.2	1.3	6.3	5.6	8.8	4.6

9.8.5. Taxa abundance with nitrogen fertiliser – Gadlas (1 week)

Top 200 OTU relative abundance (%) sequenced from soil samples taken from control plots (- N) and control plots (+ N, 25 kg ha^{1}) in June 2013 at Gadlas (GA) site.

Phylum	Class	Order	Family	Genus	- N	+ N
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	25.24	1.91
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	4.26	14.07
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	6.77	4.2
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Gamsiella	0.31	10.39
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	4.05	5.58
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	OTU 2097	6.94	0
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OTU 9	3.17	3.19
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 17	3.47	1.46
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	1.41	2.74
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	2.62	1.08
Basidiomycota	Atractiellomycetes	Atractiellales	Hoehnelomycetaceae	Atractiella	2.92	0.04
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus_ter	1.15	1.79
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 55	2.01	0.86
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Alphamycetaceae	Betamyces	1.99	0.67
X	X	X	X	OTU 11	1.05	1.59
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Massariosphaeria	1.21	1.39
Basidiomycota	Agaricomycetes	X	X	OTU 161	2.14	0.34
X	X	X	X	OTU 14	0.62	1.85
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	0.68	1.68
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Uthatobasidium	0.16	1.9
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Pyrenochaeta	0.31	1.71
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	Columnosphaeria	0.04	1.94
Ascomycota	X	X	X	OTU 5	0.36	1.47
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Lipomyces_tetrasporus	1.37	0.33
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Davidiella	0	1.66

Ascomycota	Laboulbeniomycetes	Pyxidiophorales	Pyxidiophoraceae	Pyxidiophora	1.32	0.28
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	0.33	1.21
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	OTU 38	0.07	1.45
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Graddonia	0.21	1.19
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	OTU 19	0.89	0.51
Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	uncultured_Thelephoraceae	1.22	0.16
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	0.25	1.03
Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	Ascobolus	0.81	0.43
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium complex	0.1	1.1
X	X	X	X	OTU 10294	1.16	0
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinellus	0.02	1.08
X	X	X	X	OTU 1783	0.57	0.48
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 164	0.88	0.17
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lachnum	0.03	1
Basidiomycota	Microbotryomycetes	Microbotryomycetes incertae sedis	Microbotryomycetes incertae sedis	Kriegeria	0.38	0.61
Ascomycota	X	X	X	OTU 25	0	0.96
Basidiomycota	Agaricomycetes	Auriculariales	Exidiaceae	Endoperplexa	0.02	0.94
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Piriformospora	0.22	0.59
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Panaeolus	0.34	0.46
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Mollisia	0.37	0.43
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	OTU 2550	0	0.76
Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	Sporobolomyces	0.49	0.26
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 71	0.52	0.2
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Tetracladium	0.3	0.43
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiales incertae sedis	Leucosporidium	0.06	0.65
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	0.46	0.25
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	Mrakia	0.09	0.58

X	X	X	X	OTU 102	0.54	0.12
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	0.15	0.44
Glomeromycota	Glomeromycetes	Archaeosporales	Archaeosporaceae	Archaeospora	0.46	0.06
X	X	X	X	OTU 40	0.05	0.46
Ascomycota	Leotiomycetes	Helotiales	X	OTU 16	0	0.51
Basidiomycota	Agaricomycetes	Agaricales	Omphalotaceae	Omphalotus	0.48	0.02
X	X	X	X	OTU 593	0.01	0.48
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacina	0.02	0.47
Chytridiomycota	Chytridiomycetes	X	X	OTU 2230	0.26	0.18
X	X	X	X	OTU 58	0.18	0.25
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_2	0.27	0.16
Basidiomycota	Agaricomycetes	X	X	OTU 213	0.3	0.09
Chytridiomycota	Chytridiomycetes	X	X	OTU 89	0.19	0.21
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Conocybe	0.27	0.1
Ascomycota	Leotiomycetes	Helotiales	X	OTU 9163	0.07	0.3
Ascomycota	Leotiomycetes	X	X	OTU 119	0	0.36
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 6403	0.35	0
X	X	X	X	OTU 12504	0.33	0
Chytridiomycota	Chytridiomycetes	X	X	OTU 78	0.12	0.21
Ascomycota	Leotiomycetes	Helotiales	X	OTU 52	0.09	0.21
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Issatchenkia_orientalis_ATCC_6258_(T)	0.3	0
Fungi incertae sedis	Mucoromycotina	Endogonales	Endogonaceae	Endogone	0.2	0.1
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Waitea	0	0.29
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Rickenella	0.15	0.13
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Neofabraea	0.05	0.22
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	0.06	0.2
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	Operculomyces	0.05	0.2
X	X	X	X	OTU 24	0.09	0.16
Blastocladiomycota	Blastocladiomycetes	Blastocladiales	Catenariaceae	Catenomyces	0.03	0.21
Ascomycota	Leotiomycetes	Leotiomycetes incertae sedis	Leotiomycetes incertae sedis	Collophora	0	0.23
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Nolanea	0.06	0.16

Ascomycota	X	X	X	OTU 2113	0.1	0.12
Basidiomycota	Tremellomycetes	Tremellales	X	OTU 44	0	0.22
Ascomycota	Leotiomycetes	Helotiales	X	OTU 2224	0	0.22
Basidiomycota	X	X	X	OTU 155	0.2	0.02
X	X	X	X	OTU 904	0.19	0.03
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Octospora	0	0.22
Basidiomycota	Tremellomycetes	Tremellales	X	OTU 99	0	0.21
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Cudoniella	0	0.21
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe _HY2	0	0.21
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVAR	0	0.21
Ascomycota	X	X	X	OTU 9299	0.2	0
Ascomycota	Pezizomycetes	Pezizales	Ascodesmidaceae	Ascodesmis	0.16	0.03
Basidiomycota	Agaricomycetes	X	X	OTU 506	0.06	0.12
X	X	X	X	OTU 1747	0.12	0.07
Ascomycota	Dothideomycetes	Microthyriales	Microthyriaceae	Microthyrium	0.07	0.09
Ascomycota	Ascomycota incertae sedis	Ascomycota incertae sedis	Ascomycota incertae sedis	Troposporella	0.04	0.12
X	X	X	X	OTU 108	0.09	0.07
Basidiomycota	Agaricomycetes	X	X	OTU 2254	0.11	0.05
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Septoria	0.14	0.01
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVAC	0	0.15
X	X	X	X	OTU 253	0.03	0.12
Ascomycota	X	X	X	OTU 755	0.13	0.02
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 2612	0	0.14
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	Lobulomyces	0.09	0.05
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 69	0	0.14
X	X	X	X	OTU 142	0.09	0.04
Ascomycota	X	X	X	OTU 67	0.09	0.04
Chytridiomycota	Chytridiomycetes	Cladochytriales	Nowakowskiellaceae	Nowakowskiella	0.06	0.07
Ascomycota	Leotiomycetes	X	X	OTU 3563	0.13	0
Basidiomycota	Exobasidiomycetes	Georgefischeriales	Tilletiariaceae	Tilletiaria	0.06	0.07
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Pseudombrophila	0.1	0.03
Ascomycota	X	X	X	OTU 246	0.09	0.03
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus	0.03	0.09
X	X	X	X	OTU 141	0.04	0.08

Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Saccharomyces_kudriavzevii	0	0.12
X	X	X	X	OTU 1832	0.1	0.01
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_3	0.03	0.09
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 197	0.02	0.09
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 2715	0.08	0.02
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Sorocybe	0	0.1
Basidiomycota	Agaricomycetes	X	X	OTU 1735	0.05	0.05
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_4	0.08	0.03
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 120	0.03	0.07
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Funneliformis	0.02	0.08
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus	0.03	0.08
Ascomycota	X	X	X	OTU 218	0.04	0.06
Basidiomycota	Classiculomycetes	Classiculales	Classiculaceae	Classicula	0.06	0.04
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 663	0.04	0.06
X	X	X	X	OTU 146	0.04	0.05
X	X	X	X	OTU 2225	0.01	0.09
Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	Saccobolus	0.02	0.07
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Melastiza	0.04	0.05
Basidiomycota	Pucciniomycetes	Platygloeales	Eocronartiaceae	Eocronartium	0	0.09
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Monilinia	0	0.09
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 533	0.06	0.03
X	X	X	X	OTU 169	0.05	0.03
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Torrendiella	0.08	0.01
Ascomycota	X	X	X	OTU 163	0.06	0.02
Chytridiomycota	Chytridiomycetes	Rhizophydiales	X	OTU 519	0.01	0.07
Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	Boudiera	0.04	0.05
Ascomycota	X	X	X	OTU 121	0.05	0.03
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 150	0.02	0.06
Ascomycota	X	X	X	OTU 529	0	0.08
Ascomycota	X	X	X	OTU 760	0.03	0.05
Ascomycota	Dothideomycetes	Pleosporales	Lophiostomataceae	OTU 569	0.04	0.03

Basidiomycota	Agaricostilbomycetes	Spiculogloeales	Spiculogloeales incertae sedis	Mycogloea	0.01	0.07
Basidiomycota	Atractiellomycetes	Atractiellales	X	OTU 317	0	0.07
Basidiomycota	Agaricomycetes	Boletales	Stephanosporaceae	Stephanospora	0.04	0.04
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 310	0.03	0.04
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 919	0.07	0
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 173	0.05	0.02
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	OTU 313	0.03	0.03
X	X	X	X	OTU 110	0.01	0.05
Ascomycota	Leotiomycetes	Helotiales	X	OTU 9354	0.05	0.01
Basidiomycota	Agaricomycetes	X	X	OTU 1752	0.05	0.01
X	X	X	X	OTU 126	0.04	0.02
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Eupenicillium	0.04	0.02
Ascomycota	Leotiomycetes	Helotiales	Rutstroemiaceae	Lambertella	0	0.05
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Lamprospora	0	0.06
Ascomycota	Leotiomycetes	Helotiales	X	OTU 83	0.05	0.01
X	X	X	X	OTU 761	0.03	0.02
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Chalara	0.02	0.03
X	X	X	X	OTU 284	0.01	0.05
Basidiomycota	Agaricostilbomycetes	Agaricostilbales	Chionosphaeraceae	Kurtzmanomyces	0.01	0.04
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	0	0.05
Ascomycota	X	X	X	OTU 404	0	0.05
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 387	0.03	0.02
Ascomycota	Leotiomycetes	X	X	OTU 97	0.05	0
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OTU 3238	0	0.05
X	X	X	X	OTU 379	0.03	0.02
X	X	X	X	OTU 289	0	0.05
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	Trechispora	0.03	0.02
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	OTU 376	0	0.04
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Byssonectria	0.04	0
X	X	X	X	OTU 1786	0	0.04
Ascomycota	Leotiomycetes	Helotiales	X	OTU 3442	0.04	0

Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Clitopilus	0.04	0
Basidiomycota	Agaricomycetes	Hymenochaetales	Schizoporaceae	Lagarobasidium	0.02	0.02
Ascomycota	Leotiomycetes	X	X	OTU 491	0.02	0.02
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_2	0.01	0.03
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	Melanomma	0	0.04
X	X	X	X	OTU 441	0	0.04
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Amylomyces	0	0.04
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	OTU 205	0	0.04
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Hamigera	0.02	0.01
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavulinopsis_CPLA	0	0.03
Ascomycota	X	X	X	OTU 11176	0.03	0
Ascomycota	X	X	X	OTU 534	0	0.03
X	X	X	X	OTU 627	0.03	0
X	X	X	X	OTU 1754	0.02	0.01
X	X	X	X	OTU 1553	0.02	0.01
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	Maunachytrium	0.01	0.02
Basidiomycota	Agaricomycetes	Agaricomycetes incertae sedis	Agaricomycetes incertae sedis	Tricellulortus	0.03	0
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe _PS1	0	0.03
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVX1	0.01	0.02
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Ceratobasidium	0	0.02
X	X	X	X	OTU 602	0.02	0.01
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 894	0	0.03

9.8.6. Taxa abundance with nitrogen fertiliser – Gadlas (July)

Top 200 OTU relative abundance (%) sequenced from soil samples taken from buffer plots (- N) and control plots (+ N, 50 kg ha^{1}) in July 2013 at Gadlas (GA) site.

Phylum	Class	Order	Family	Genus	- N	+ N
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	15.01	1.04
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	12.18	2.57
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	6.11	6.97
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 55	4.58	5.72
Basidiomycota	Tremellomycetes	Tremellales	X	OTU 99	0.40	7.22
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Pyrenochaeta	2.61	4.77
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	3.95	2.85
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	1.10	4.37
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium complex	0.49	4.67
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OTU 9	1.83	1.84
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	1.46	1.91
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Gamsiella	0.06	3.06
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Waitea	3.09	0.37
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 17	1.64	1.58
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 2612	2.91	0.00
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Graddonia	1.35	1.26
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Mollisia	2.05	0.61
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	1.80	0.79
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus_ter	0.89	1.53
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Piriformospora	1.79	0.73
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	Mrakia	0.28	1.75
X	X	X	X	OTU 11	0.61	1.31
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacina	0.93	1.04
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Issatchenkia_orientalis_ATCC_6258_(T)	2.16	0.00
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Massariosphaeria	1.14	0.74
Basidiomycota	Agaricomycetes	X	X	OTU 161	0.64	1.06

Basidiomycota	Microbotryomycetes	X	X	OTU 748	0.21	1.38
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 69	0.49	1.13
Ascomycota	Leotiomycetes	Helotiales	X	OTU 52	0.25	1.33
Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	Ascobolus	0.89	0.80
Ascomycota	X	X	X	OTU 5	0.17	1.27
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	OTU 19	0.61	0.87
Basidiomycota	X	X	X	OTU 6068	0.00	1.32
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	1.22	0.24
Ascomycota	Leotiomycetes	Helotiales	X	OTU 83	1.34	0.11
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Alphamycetaceae	Betamyces	0.88	0.46
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	OTU 38	0.00	1.19
Chytridiomycota	Chytridiomycetes	X	X	OTU 89	0.00	1.18
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	0.31	0.85
Glomeromycota	Glomeromycetes	Archaeosporales	Archaeosporaceae	Archaeospora	0.56	0.53
X	X	X	X	OTU 102	0.78	0.27
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	0.41	0.53
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Rickenella	0.65	0.32
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Pilidium	0.98	0.03
Ascomycota	Leotiomycetes	Helotiales	X	OTU 16	0.29	0.54
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Tetracladium	0.39	0.44
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_2	0.63	0.22
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Lipomyces_tetrasporus	0.40	0.38
X	X	X	X	OTU 1783	0.23	0.53
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	OTU 11215	0.80	0.00
Ascomycota	Leotiomycetes	Helotiales	X	OTU 2224	0.27	0.42
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus	0.65	0.11
Basidiomycota	Microbotryomycetes	Microbotryales	Ustilentylomataceae	Ustilentyloma	0.13	0.52
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	Operculomyces	0.44	0.24
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	OTU 894	0.72	0.00
Basidiomycota	Microbotryomycetes	Microbotryomycetes incertae sedis	Microbotryomycetes incertae sedis	Kriegeria	0.33	0.29

Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	uncultured_Thelephoraceae	0.33	0.29
Ascomycota	Leotiomycetes	X	X	OTU 97	0.15	0.44
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Ellisomyces	0.23	0.36
X	X	X	X	OTU 1747	0.55	0.03
Basidiomycota	X	X	X	OTU 155	0.29	0.24
Fungi incertae sedis	Mucoromycotina	Endogonales	Endogonaceae	Endogone	0.24	0.28
Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	Sporobolomyces	0.17	0.33
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 150	0.57	0.00
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Byssonectria	0.00	0.46
X	X	X	X	OTU 14	0.03	0.42
Basidiomycota	Agaricomycetes	X	X	OTU 213	0.48	0.03
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Boeremia	0.00	0.43
Ascomycota	Leotiomycetes	Helotiales	X	OTU 12624	0.00	0.43
Ascomycota	Laboulbeniomycetes	Pyxidiophorales	Pyxidiophoraceae	Pyxidiophora	0.14	0.30
Chytridiomycota	Chytridiomycetes	X	X	OTU 78	0.14	0.29
Basidiomycota	Tremellomycetes	Tremellales	X	OTU 11549	0.45	0.00
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	OTU 2097	0.00	0.37
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 164	0.04	0.33
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	Columnosphaeria	0.17	0.20
Basidiomycota	Agaricomycetes	Agaricomycetes incertae sedis	Agaricomycetes incertae sedis	Tricellulortus	0.15	0.19
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lachnum	0.25	0.09
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Panaeolus	0.05	0.25
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Conocybe	0.15	0.16
Ascomycota	Leotiomycetes	Helotiales	Rutstroemiaceae	Lambertella	0.08	0.22
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	endophyte	0.08	0.22
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_4	0.21	0.10
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Octospora	0.21	0.09
X	X	X	X	OTU 379	0.23	0.07
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiales incertae sedis	Leucosporidium	0.00	0.25
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 14953	0.00	0.24

Basidiomycota	Agaricomycetes	Agaricales	Marasmiaceae	Marasmius	0.25	0.02
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 197	0.00	0.23
X	X	X	X	OTU 108	0.23	0.04
X	X	X	X	OTU 15986	0.00	0.22
X	X	X	X	OTU 904	0.12	0.12
X	X	X	X	OTU 761	0.18	0.05
Blastocladiomycota	Blastocladiomycetes	Blastocladiales	Catenariaceae	Catenomyces	0.10	0.11
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OTU 2627	0.23	0.00
Ascomycota	Ascomycota incertae sedis	Ascomycota incertae sedis	Ascomycota incertae sedis	Troposporella	0.09	0.12
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Clathrosporium	0.00	0.19
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Melastiza	0.09	0.10
X	X	X	X	OTU 253	0.00	0.18
Ascomycota	Leotiomycetes	X	X	OTU 3563	0.21	0.00
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellales incertae sedis	Modicella	0.20	0.00
Basidiomycota	Agaricomycetes	X	X	OTU 2254	0.05	0.13
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 120	0.05	0.13
Ascomycota	X	X	X	OTU 9299	0.15	0.04
Basidiomycota	Exobasidiomycetes	Georgefischeriales	Tilletiariaceae	Tilletiaria	0.02	0.15
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 4082	0.00	0.16
Chytridiomycota	Chytridiomycetes	Cladochytriales	Nowakowskiellaceae	Nowakowskiella	0.03	0.13
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Spirosphaera	0.06	0.11
Ascomycota	Dothideomycetes	Microthyriales	Microthyriaceae	Microthyrium	0.10	0.08
Basidiomycota	Classiculomycetes	Classiculales	Classiculaceae	Classicula	0.00	0.16
Ascomycota	Leotiomycetes	Helotiales	X	OTU 5161	0.17	0.01
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Asterotremella	0.00	0.15
X	X	X	X	OTU 602	0.12	0.05
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 6403	0.18	0.00
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus	0.00	0.15
Ascomycota	Leotiomycetes	X	X	OTU 119	0.00	0.15
X	X	X	X	OTU 110	0.00	0.14
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Uthatobasidium	0.07	0.09
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 2715	0.00	0.14

Ascomycota	X	X	X	OTU 755	0.05	0.10
Ascomycota	X	X	X	OTU 760	0.10	0.06
X	X	X	X	OTU 1754	0.14	0.02
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Nolanea	0.00	0.13
Ascomycota	X	X	X	OTU 11176	0.11	0.04
X	X	X	X	OTU 1786	0.00	0.13
Ascomycota	Sordariomycetes	Sordariomycetes incertae sedis	Sordariomycetes incertae sedis	Hilberina	0.14	0.01
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 533	0.07	0.07
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_3	0.07	0.07
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 14493	0.00	0.12
X	X	X	X	OTU 40	0.00	0.12
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVX1	0.00	0.12
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Isomucor	0.00	0.11
X	X	X	X	OTU 9841	0.01	0.11
Ascomycota	Leotiomycetes	Helotiales	X	OTU 3309	0.00	0.11
Basidiomycota	Agaricomycetes	X	X	OTU 506	0.01	0.10
Ascomycota	Pezizomycetes	Pezizales	Ascodesmidaceae	Ascodesmis	0.03	0.08
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 14492	0.00	0.10
Basidiomycota	Agaricomycetes	X	X	OTU 686	0.00	0.10
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	0.02	0.09
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 14359	0.00	0.10
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Monilinia	0.06	0.05
Ascomycota	X	X	X	OTU 277	0.10	0.01
Basidiomycota	Pucciniomycetes	Platygloeales	Eocronartiaceae	Eocronartium	0.01	0.09
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 919	0.11	0.01
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 310	0.04	0.06
Glomeromycota	Glomeromycetes	Paraglomerales	Paraglomeraceae	Paraglomus	0.02	0.08
X	X	X	X	OTU 146	0.02	0.07
Ascomycota	X	X	X	OTU 67	0.02	0.07
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe _PS1	0.00	0.08
X	X	X	X	OTU 225	0.03	0.06
Basidiomycota	Agaricomycetes	X	X	OTU 1752	0.10	0.00

Basidiomycota	Agaricomycetes	Auriculariales	Exidiaceae	Endoperplexa	0.05	0.04
X	X	X	X	OTU 58	0.01	0.07
X	X	X	X	OTU 24	0.02	0.06
X	X	X	X	OTU 1832	0.08	0.01
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Pseudombrophila	0.04	0.04
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	OTU 313	0.00	0.07
X	X	X	X	OTU 142	0.04	0.04
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Neofabraea	0.00	0.07
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 71	0.08	0.00
Ascomycota	X	X	X	OTU 529	0.07	0.01
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	Trechispora	0.00	0.07
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	0.00	0.07
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 173	0.00	0.07
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	0.01	0.06
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Torrendiella	0.00	0.07
X	X	X	X	OTU 141	0.00	0.07
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 663	0.04	0.03
X	X	X	X	OTU 14254	0.00	0.06
Ascomycota	X	X	X	OTU 613	0.00	0.06
Ascomycota	Dothideomycetes	Pleosporales	Lophiostomataceae	OTU 569	0.00	0.06
Ascomycota	X	X	X	OTU 121	0.02	0.05
Basidiomycota	Agaricomycetes	X	X	OTU 2477	0.01	0.05
Basidiomycota	Atractiellomycetes	Atractiellales	Hoehnelomycetaceae	Atractiella	0.01	0.05
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	OTU 3067	0.07	0.00
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	Lobulomyces	0.02	0.04
Ascomycota	X	X	X	OTU 178	0.00	0.06
Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	Boudiera	0.04	0.03
Basidiomycota	Agaricomycetes	X	X	OTU 14499	0.00	0.06
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinellus	0.00	0.06
Ascomycota	Dothideomycetes	Pleosporales	Lentitheciaceae	Keissleriella	0.04	0.02
Basidiomycota	Agaricostilbomycetes	Spiculogloeales	Spiculogloeales incertae sedis	Mycogloea	0.00	0.05
Ascomycota	X	X	X	OTU 163	0.00	0.05
Ascomycota	X	X	X	OTU 721	0.02	0.04

Ascomycota	X	X	X	OTU 246	0.00	0.05
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	Melanomma	0.02	0.03
Chytridiomycota	Chytridiomycetes	Rhizophydiales	X	OTU 519	0.01	0.04
X	X	X	X	OTU 1553	0.01	0.04
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	OTU 2607	0.00	0.05
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Davidiella	0.00	0.04
Basidiomycota	Agaricostilbomycetes	Agaricostilbales	Chionosphaeraceae	Kurtzmanomyces	0.03	0.02
Basidiomycota	Agaricomycetes	X	X	OTU 2163	0.00	0.04
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeosphaeria	0.00	0.04
X	X	X	X	OTU 284	0.00	0.04
Ascomycota	X	X	X	OTU 404	0.00	0.04
X	X	X	X	OTU 593	0.00	0.04
Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	OTU 2718	0.03	0.01
X	X	X	X	OTU 12504	0.00	0.04
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_2	0.00	0.04
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Flagelloscypha	0.03	0.01
Basidiomycota	Agaricomycetes	Agaricales	Omphalotaceae	Omphalotus	0.00	0.03

9.8.7. Taxa abundance with nitrogen fertiliser – Gadlas (Sept.)

Top 200 OTU relative abundance (%) sequenced from soil samples taken from buffer plots (- N) and control plots (+ N, 100 kg ha^{1}) in September 2013 at Gadlas (GA) site.

Phylum	Class	Order	Family	Genus	- N	+ N
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Pyrenochaeta	15.28	8.39
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	6.57	7.20
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	10.19	2.50
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	7.29	1.62
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	2.93	4.84
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 55	4.21	3.07
Ascomycota	Leotiomycetes	Helotiales	X	OTU 16	4.03	1.08
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	OTU 19	2.74	2.54
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	1.68	3.25
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Gamsiella	0.03	4.84
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 69	2.67	1.28
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OTU 3238	3.25	0.00
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OTU 9	1.32	2.26
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium complex	1.06	2.33
Ascomycota	X	X	X	OTU 5	1.40	1.67
X	X	X	X	OTU 11	0.64	2.09
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	OTU 12516	0.00	2.60
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Massariosphaeria	0.70	1.68
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 17	0.91	1.39
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	0.87	1.41
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Piriformospora	1.84	0.17
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	0.51	1.72
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	0.55	1.64
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Flagelloscypha	1.75	0.02
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus_ter	0.35	1.54

Chytridiomycota	Chytridiomycetes	Rhizophydiales	Alphamycetaceae	Betamyces	0.69	0.86
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Tetracladium	0.47	1.05
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	OTU 14717	0.00	1.48
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lachnum	0.57	0.63
Basidiomycota	Agaricomycetes	X	X	OTU 161	0.29	0.94
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeosphaeria	0.05	1.23
Basidiomycota	X	X	X	OTU 155	0.83	0.21
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	0.18	0.97
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Mollisia	0.48	0.55
Fungi incertae sedis	Mucoromycotina	Endogonales	Endogonaceae	Endogone	0.67	0.30
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacina	0.47	0.54
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Lipomyces_tetrasporus	0.36	0.64
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Graddonia	0.59	0.36
Basidiomycota	Agaricomycetes	X	X	OTU 13578	0.84	0.00
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Ophiosphaerella	0.69	0.03
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_2	0.24	0.52
Basidiomycota	Pucciniomycetes	Platygloeales	Eocronartiaceae	Eocronartium	0.62	0.06
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	OTU 6936	0.30	0.42
X	X	X	X	OTU 102	0.41	0.28
Ascomycota	Laboulbeniomycetes	Pyxidiophorales	Pyxidiophoraceae	Pyxidiophora	0.01	0.76
Ascomycota	Leotiomycetes	X	X	OTU 97	0.00	0.77
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus	0.43	0.25
Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	Sporobolomyces	0.09	0.59
Ascomycota	Leotiomycetes	X	X	OTU 16	0.57	0.00
Chytridiomycota	Chytridiomycetes	X	X	OTU 78	0.25	0.38
Basidiomycota	Microbotryomycetes	Microbotryomycetes incertae sedis	Microbotryomycetes incertae sedis	Kriegeria	0.11	0.55
Basidiomycota	Atractiellomycetes	Atractiellales	Hoehnelomycetaceae	Atractiella	0.08	0.58
Ascomycota	Leotiomycetes	Helotiales	X	OTU 52	0.12	0.53
Basidiomycota	X	X	X	OTU 8696	0.51	0.02
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Torrendiella	0.37	0.18
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 14053	0.52	0.00
Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	Ascobolus	0.18	0.37

Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Ceratobasidium	0.34	0.18
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	0.36	0.14
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus_4	0.26	0.25
Basidiomycota	Agaricomycetes	X	X	OTU 13566	0.41	0.01
Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	uncultured_Thelephoraceae	0.32	0.11
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Rickenella	0.39	0.02
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 14051	0.40	0.00
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Conocybe	0.35	0.06
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_2	0.17	0.27
X	X	X	X	OTU 1747	0.20	0.23
Ascomycota	Dothideomycetes	Capnodiales	Dissoconiaceae	Dissoconium	0.13	0.31
Glomeromycota	Glomeromycetes	Paraglomerales	Paraglomeraceae	Paraglomus	0.16	0.27
Ascomycota	Leotiomycetes	Helotiales	X	OTU 705	0.00	0.46
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	Melanomma	0.37	0.01
Glomeromycota	Glomeromycetes	X	X	OTU 6805	0.20	0.19
Basidiomycota	Agaricostilbomycetes	Agaricostilbales	Chionosphaeraceae	Kurtzmanomyces	0.31	0.05
Ascomycota	Dothideomycetes	Pleosporales	Lophiostomataceae	OTU 569	0.32	0.04
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Panaeolus	0.11	0.27
X	X	X	X	OTU 1783	0.08	0.28
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 14482	0.00	0.38
Ascomycota	X	X	X	OTU 9299	0.17	0.17
Basidiomycota	Classiculomycetes	Classiculales	Classiculaceae	Classicula	0.02	0.34
Basidiomycota	Exobasidiomycetes	Georgefischeriales	Tilletiariaceae	Tilletiaria	0.02	0.32
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Neofabraea	0.28	0.00
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	OTU 38	0.11	0.20
Basidiomycota	Agaricomycetes	Agaricomycetes incertae sedis	Agaricomycetes incertae sedis	Tricellulortus	0.25	0.04
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Cudoniella	0.00	0.33
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	0.03	0.29
X	X	X	X	OTU 126	0.23	0.04
X	X	X	X	OTU 110	0.01	0.29
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	Columnosphaeria	0.04	0.25
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 919	0.23	0.01

Basidiomycota	Agaricomycetes	Agaricales	Omphalotaceae	Omphalotus	0.07	0.21
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 2282	0.07	0.20
Chytridiomycota	Chytridiomycetes	X	X	OTU 536	0.23	0.00
Fungi incertae sedis	Mucoromycotina	Mucorales	Mucoraceae	Mucor	0.02	0.25
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Uthatobasidium	0.09	0.16
Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus	0.10	0.15
Ascomycota	X	X	X	OTU 613	0.10	0.15
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 197	0.00	0.27
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	OTU 2097	0.01	0.26
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Pilidium	0.21	0.01
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Clathrosporium	0.07	0.18
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 533	0.13	0.10
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	OTU 11215	0.21	0.00
Basidiomycota	Agaricomycetes	Hymenochaetales	X	OTU 164	0.02	0.23
X	X	X	X	OTU 9841	0.20	0.00
Basidiomycota	Agaricomycetes	X	X	OTU 14089	0.19	0.01
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Nolanea	0.01	0.22
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Pseudombrophila	0.05	0.16
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	OTU 14493	0.00	0.22
Ascomycota	Leotiomycetes	Helotiales	X	OTU 83	0.05	0.16
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	Operculomyces	0.07	0.13
Ascomycota	Ascomycota incertae sedis	Ascomycota incertae sedis	Ascomycota incertae sedis	Troposporella	0.08	0.11
Ascomycota	Leotiomycetes	Helotiales	X	OTU 5161	0.10	0.08
Ascomycota	X	X	X	OTU 404	0.01	0.18
X	X	X	X	OTU 904	0.03	0.16
X	X	X	X	OTU 593	0.15	0.01
Chytridiomycota	Chytridiomycetes	Cladochytriales	Nowakowskiellaceae	Nowakowskiella	0.06	0.12
Basidiomycota	Agaricomycetes	X	X	OTU 2477	0.15	0.00
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 663	0.08	0.08
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Clitopilus	0.14	0.00

Basidiomycota	Agaricomycetes	Thelephorales	X	OTU 16797	0.00	0.17
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVX1	0.08	0.07
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Candida_sake_(T)	0.13	0.00
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 71	0.13	0.00
Ascomycota	Pezizomycetes	Pezizales	Ascodesmidaceae	Ascodesmis	0.04	0.11
Basidiomycota	Agaricostilbomycetes	Spiculogloeales	Spiculogloeales incertae sedis	Mycogloea	0.03	0.12
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OTU 2627	0.01	0.14
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 120	0.01	0.15
X	X	X	X	OTU 225	0.10	0.03
Glomeromycota	Glomeromycetes	Archaeosporales	Archaeosporaceae	Archaeospora	0.08	0.06
Basidiomycota	Agaricomycetes	X	X	OTU 14225	0.00	0.15
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 2715	0.02	0.12
Ascomycota	Dothideomycetes	Pleosporales	X	OTU 11426	0.10	0.03
Ascomycota	X	X	X	OTU 121	0.02	0.12
Chytridiomycota	Chytridiomycetes	X	X	OTU 89	0.00	0.14
Ascomycota	Leotiomycetes	Helotiales	Rutstroemiaceae	Lambertella	0.05	0.08
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Boeremia	0.00	0.14
Ascomycota	X	X	X	OTU 178	0.07	0.06
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 14953	0.00	0.14
Ascomycota	X	X	X	OTU 67	0.00	0.13
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	endophyte	0.07	0.04
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Funneliformis	0.02	0.11
Basidiomycota	Agaricomycetes	Trechisporales	Trechisporaceae	Trechispora	0.01	0.11
Basidiomycota	Agaricomycetes	Hymenochaetales	Schizoporaceae	Lagarobasidium	0.00	0.12
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Spirosphaera	0.08	0.03
X	X	X	X	OTU 146	0.00	0.12
X	X	X	X	OTU 142	0.02	0.09
Basidiomycota	Agaricomycetes	X	X	OTU 2254	0.02	0.09
X	X	X	X	OTU 627	0.09	0.00
X	X	X	X	OTU 1786	0.00	0.11
Glomeromycota	Glomeromycetes	Diversisporales	Gigasporaceae	Scutellospora	0.06	0.03
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 14492	0.00	0.11

Basidiomycota	Tremellomycetes	Tremellales	X	OTU 99	0.07	0.03
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_3	0.01	0.10
X	X	X	X	OTU 14219	0.00	0.11
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Parasola	0.08	0.00
Chytridiomycota	Chytridiomycetes	Rhizophydiales	X	OTU 14879	0.00	0.10
Ascomycota	X	X	X	OTU 277	0.05	0.04
X	X	X	X	OTU 1754	0.04	0.05
Ascomycota	Leotiomycetes	Helotiales	X	OTU 5734	0.07	0.01
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Calyptella	0.08	0.00
Ascomycota	Pezizomycetes	Pezizales	Ascobolaceae	Saccobolus	0.01	0.08
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	OTU 313	0.01	0.08
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Waitea	0.07	0.01
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	0.00	0.09
Ascomycota	X	X	X	OTU 755	0.02	0.07
Basidiomycota	Agaricomycetes	Auriculariales	Exidiaceae	Endoperplexa	0.06	0.02
X	X	X	X	OTU 253	0.00	0.08
X	X	X	X	OTU 602	0.04	0.04
Ascomycota	Leotiomycetes	X	X	OTU 491	0.00	0.08
Ascomycota	Dothideomycetes	Microthyriales	Microthyriaceae	Microthyrium	0.02	0.06
X	X	X	X	OTU 14254	0.00	0.08
Ascomycota	Dothideomycetes	Pleosporales	Lentitheciaceae	Keissleriella	0.04	0.03
Basidiomycota	Agaricomycetes	X	X	OTU 14499	0.00	0.07
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	OTU 173	0.01	0.06
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Eupenicillium	0.02	0.05
X	X	X	X	OTU 14	0.00	0.07
Chytridiomycota	Chytridiomycetes	Rhizophydiales	X	OTU 519	0.02	0.04
Ascomycota	X	X	X	OTU 163	0.04	0.02
Ascomycota	Sordariomycetes	Sordariomycetes incertae sedis	Sordariomycetes incertae sedis	Hilberina	0.02	0.04
Ascomycota	X	X	X	OTU 11176	0.02	0.04
Basidiomycota	Agaricomycetes	X	X	OTU 686	0.00	0.07
Chytridiomycota	Chytridiomycetes	Lobulomycetales	Lobulomycetaceae	Lobulomyces	0.02	0.04
Ascomycota	Leotiomycetes	X	X	OTU 119	0.00	0.07
Chytridiomycota	Chytridiomycetes	Rhizophydiales	Rhizophydiales incertae sedis	OTU 14359	0.00	0.06
X	X	X	X	OTU 141	0.00	0.06

Ascomycota	Eurotiomycetes	Onygenales	Arachnomycetaceae	Arachnomyces	0.02	0.03
Basidiomycota	Agaricomycetes	Agaricales	Marasmiaceae	Marasmius	0.01	0.05
Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	Boudiera	0.00	0.05
X	X	X	X	OTU 108	0.03	0.02
X	X	X	X	OTU 58	0.04	0.00
Ascomycota	X	X	X	OTU 246	0.00	0.04
Ascomycota	X	X	X	OTU 218	0.02	0.02
Basidiomycota	X	X	X	OTU 14933	0.00	0.04
X	X	X	X	OTU 1832	0.03	0.01
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Lamprospora	0.03	0.00
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	OTU 4082	0.00	0.04
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	OTU 2607	0.00	0.04
Ascomycota	Dothideomycetes	Pleosporales	Montagnulaceae	Bimuria	0.00	0.04
Ascomycota	Leotiomycetes	X	X	OTU 3563	0.03	0.00
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 2612	0.03	0.00
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	OTU 205	0.00	0.04

9.8.8. Phylum abundance - P-index

Table showing the phyla abundance of all plots, Control and BI treated (statistical analysis had found no significant effect of BI on any parameters with increasing P-index). Plant-available P had no effect on fungal phyla abundance or diversity measures (Anova).

	P-index			
Fungal phyla abundance (%)	**0** (*n* = 5)	**1** (*n* = 6)	**2** (*n* = 4)	**3** (*n* = 3)
Ascomycota	49.59 (± 17.6)	51.79 (± 18.9)	50.97 (± 16.3)	46.42 (± 4.1)
Basidiomycota	39.76 (± 16.7)	40.36 (± 19.6)	41.01 (± 12.5)	44.38 (± 6.9)
Blastocladiomycota	0.01 (± 0.01)	0.03 (± 0.03)	0.00	0.01 (± 0.01)
Chytridiomycota	1.18 (± 0.04)	0.80 (± 0.3)	0.76 (± 0.4)	1.44 (± 0.9)
Fungi incertae sedis	3.97 (± 1.5)	2.44 (± 1.7)	2.37 (± 1.2)	2.54 (± 2.2)
Glomeromycota	1.45 (± 0.4)	1.34 (± 0.8)	1.10 (± 0.5)	1.48 (± 0.4)
OTU	528 (± 105)	297 (± 132)	400 (± 48)	316 (± 83)
Shannon index (H)	4.00 (± 0.35)	3.72 (± 0.40)	3.91 (± 0.15)	4.18 (± 0.15)
Shannon equitability (E_H)	0.64 (± 0.04)	0.67 (± 0.05)	0.65 (± 0.03)	0.73 (± 0.01)

9.8.9. Phylum abundance - BI treatment (Gadlas)

Effects of bio-inoculant (BI) treatment at two application rates (Recommended (Rec.) and ten times recommended (×10)) on abundances of fungal phyla (%), diversity (*H*) and equitability (E_H) (based on operational taxonomic unit counts (OTUs)) at the Gadlas (GA) site. Values in parenthesis are ± 1 standard deviation.

Fungal phyla abundance (%)	**Control**	**Biagro® Grass**		**Biagro® MP**		**Biagro® S**	
		Rec.	**×10**	**Rec.**	**×10**	**Rec.**	**×10**
Ascomycota	35.51 (± 15.42)	47.91 (± 14.57)	51.43 (± 6.93)	68.22 (± 2.77)	43.85 (± 21.98)	45.13 (± 16.06)	36.92 (± 4.70)
Basidiomycota	25.80 (± 10.41)	27.20 (± 14.89)	15.26 (± 7.66)	16.59 (± 3.61)	16.24 (± 6.92)	18.65 (± 6.93)	22.70 (± 15.58)
Blastocladiomycota	0.03 (± 0.04)	0.05 (± 0.09)	0.02 (± 0.02)	0.03 (± 0.04)	0.33 (± 0.29)	0.00	3.61 (± 5.90)
Chytridiomycota	4.10 (± 2.26)	3.16 (± 1.80)	3.76 (± 0.91)	2.25 (± 0.74)	8.67 (± 2.52)	3.48 (± 1.81)	5.92 (± 2.53)
Fungi incertae sedis	25.97 (± 4.99)	14.09 (± 2.90)	18.83 (± 2.81)	7.49 (± 3.56)	22.67 (± 14.49)	22.96 (± 9.28)	21.13 (± 6.48)
Glomeromycota	1.39 (± 1.02)	0.75 (± 0.65)	0.38 (± 0.25)	0.28 (± 0.01)	0.74 (± 0.74)	0.75 (± 0.70)	0.66 (± 0.59)
Diversity							
Shannon index (H)	3.22 (± 0.49)	3.22 (± 0.29)	3.66 (± 0.46)	3.62 (± 0.37)	3.20 (± 0.24)	3.31 (± 0.27)	3.58 (± 0.46)
Shannon equitability (E_H)	0.63 (± 0.03)	0.65 (± 0.04)	0.67 (± 0.06)	0.67 (± 0.10)	0.64 (± 0.03)	0.66 (± 0.03)	0.67 (± 0.01)

9.8.10. Phylum abundance - BI treatment (Morfa Ganol)

Effects of bio-inoculant (BI) treatment on abundances of fungal phyla (%), diversity (*H*) and equitability (E_H) (based on operational taxonomic unit counts (OTUs)) at the Morfa Ganol (MG) site. Where there was a significant treatment effect, different superscript letters indicate significantly different means (Kruskal-Wallis, $p < 0.05$). Values in parenthesis are ± 1 standard deviation.

Fungal phyla abundance (%)	**Control**	**Biagro® Grass**	**SSI**	**Biagro® PhosN**
Ascomycota	47.31 (± 14.30)	55.99 (± 16.82)	57.51 (± 17.36)	65.04 (± 13.36)
Basidiomycota	46.69 (± 14.60)	36.92 (± 17.12)	36.90 (± 17.22)	30.34 (± 12.80)
Chytridiomycota	0.72 (± 0.39)	0.83 (± 0.33)	0.62 (± 0.58)	0.71 (± 0.35)
Fungi incertae sedis	2.78^{ab} (± 2.20)	3.77^{b} (± 2.80)	3.02^{ab} (± 1.40)	1.29^{b} (± 0.54)
Glomeromycota	0.74 (± 0.30)	0.83 (± 0.37)	0.55 (± 0.18)	0.76 (± 0.35)
Diversity				
Shannon index (H)	3.05 (± 0.46)	3.02 (± 0.44)	3.03 (± 0.24)	3.29 (± 0.23)
Shannon equitability (E_H)	0.70 (± 0.09)	0.70 (± 0.10)	0.71 (± 0.05)	0.74 (± 0.04)

9.8.11. Sequenced BIs

Each BI individually sequenced. Twenty of the most abundant fungi found are given (%).

Biagro® Grass

Phylum	***Class***	***Order***	***Family***	***Genus***	**%**
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	43.73
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	11.13
Ascomycota	Eurotiomycetes	Onygenales	Arachnomycetaceae	Arachnomyces	10.48
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Eupenicillium	3.76
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	2.53
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	1.15
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinopsis	0.83
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	endophyte	0.75
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe _HY2	0.39
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_3	0.39
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Tetracladium	0.36
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus_CU4	0.36
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	0.35
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Sorocybe	0.32
Basidiomycota	Atractiellomycetes	Atractiellales	X	OTU 317	0.29
Ascomycota	Leotiomycetes	Leotiomycetes incertae sedis	Leotiomycetes incertae sedis	Collophora	0.27
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Davidiella	0.25
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Godronia	0.23
Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	uncultured_Thelephoraceae	0.23
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	0.17

Biagro® MP

Phylum	*Class*	*Order*	*Family*	*Genus*	*%*
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Saccharomyces	77.18
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	1.39
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	1.26
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	1.07
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus_CU4	0.55
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Nolanea	0.47
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	OTU 17	0.39
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus_ter	0.35
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Issatchenkia_orientalis	0.33
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Uthatobasidium	0.30
Ascomycota	Dothideomycetes	Pleosporales	Pleosporales incertae sedis	Massariosphaeria	0.30
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	Mrakia	0.30
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	0.27
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Cryptococcus	0.25
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	OTU 3382	0.25
Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Piriformospora	0.23
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	0.21
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe _HY2	0.20
Basidiomycota	Agaricomycetes	Agaricales	X	OTU 69	0.19
Ascomycota	Laboulbeniomycetes	Pyxidiophorales	Pyxidiophoraceae	Pyxidiophora	0.18

Biagro® PhosN

Phylum	*Class*	*Order*	*Family*	*Genus*	*%*
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Geotrichum_silvicola	58.70
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Issatchenkia_orientalis_ATCC_6258_(T)	6.84
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	OTU 3408	0.65
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Ceratobasidium	0.45
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Neofabraea	0.36
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	0.24
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lachnum	0.09
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Hyaloscypha	0.07
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	0.04
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	0.04
Basidiomycota	Agaricomycetes	Hymenochaetales	Schizoporaceae	Lagarobasidium	0.04
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus_CU4	0.03
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe _PS1	0.03
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Thanatephorus	0.03
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	Saccharomyces_kudriavzevii	0.02
Ascomycota	Leotiomycetes	Helotiales	X	OTU 12624	0.02
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	0.02
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Sorocybe	0.02
Ascomycota	Pezizomycetes	Pezizales	Ascodesmidaceae	Ascodesmis	0.01
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVAC	0.01

Single MF inoculant

Phylum	*Class*	*Order*	*Family*	*Genus*	*%*
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	endophyte	18.52
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	12.20
Ascomycota	Leotiomycetes	Helotiales	Helotiales incertae sedis	Tetracladium	2.35
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	1.83
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus_3	1.78
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium complex	1.65
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Cuphophyllus_CU4	1.51
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	1.46
Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe _HY2	1.33
Basidiomycota	Atractiellomycetes	Atractiellales	X	OTU 317	1.30
Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVAC	1.26
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	Thelebolus	1.26
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Veronaea	1.15
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Sorocybe	1.04
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	1.03
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	0.89
Fungi incertae sedis	Mortierellomycotina	Mortierellales	Mortierellaceae	Mortierella	0.78

Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria_CVAR	0.75
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lachnum	0.67
Ascomycota	Eurotiomycetes	Verrucariales	Verrucariaceae	OTU 2056	0.66