Linking plant growth promoting arbuscular mycorrhizal colonization with bacterial plant sulfur supply

Authors: Jacinta Gahan, and Achim Schmalenberger

5

Department of Biological Sciences, School of Natural Sciences, University of Limerick,
Limerick, Ireland

10 Corresponding author*: Achim Schmalenberger,
Department of Biological Sciences,
University of Limerick,
Park Road, Castletroy,
Limerick, V94T9PX,
15 Rep. of Ireland

Abstract

Sulfur (S) exists in organically bound complexes (~95%), predominantly as sulfonates, and are not directly plant available. Specific soil bacteria can mobilise sulfonates but very little is known about these bacteria in the hyphosphere. Since mycorrhizal fungi support growth of the majority of land plants, hyphosphere desulfonating bacteria may be of substantial benefit to the plant host. This study analysed the effect of AM inoculation with *Rhizophagus irregularis* (former *G. intraradices*, Glomus) and a mix of six AM species (Mixed) on PGP, microbial communities and sulfonate mobilising bacteria with *L. perenne*, *Agrostis stolonifera* and *Plantago lanceolata* as plant hosts in bi-compartmental microcosms and *A. stolonifera* in PGP pot experiments. AM inoculation significantly increased plant growth, percentage root colonisation and the quantity of cultivable desulfonating bacteria in the hyphosphere over pre-inoculated soil for all plants. Community analysis via PCR-DGGE revealed significantly different bacterial and fungal communities post inoculation. Analysis of the sulfonate mobilising *asfA* gene revealed a significantly altered community and novel bacterial isolates with this important functional ability post-inoculation. The results demonstrate that AM inoculation increased plant biomass yield, AM root colonisation and altered bacterial and fungal community dynamics in the hyphosphere. AM inoculated microcosms had an increased abundance of desulfonating bacteria that may be beneficial for plant-S supply.

Research highlights

- 40 • Inoculation with AM fungi was shown to promote plant growth and harbour larger populations of sulfonate mobilising bacteria.
- Post-inoculation hyphospheric bacterial and saprotrophic fungal communities were shown to differ significantly in composition and abundance.
- 45 • Analysis of sulfonate mobilising bacteria revealed novel presumptive species in possession of the *asfA* gene associated with AM hyphae.
- AM inoculation was shown to significantly impact the *asfA* positive bacterial community composition.

50 **1. Introduction**

Agricultural land has been subjected to human induced degradation in recent years as a result of intensification of agricultural practices leading to ecosystem alterations including soil degradation and erosion (Barrios, 2007). Restoration and sustainable management of agricultural land is a necessity given the growing global population, 55 estimated to double by 2050, and increased demand from agricultural land projected to be 60-110% of the current rate (Ray *et al.*, 2013).

Arbuscular Mycorrhizal (AM) fungi are ubiquitous soil microorganisms that form endo-symbiotic partnerships with most land plants. They exist in almost all terrestrial ecosystems where they may be involved in sustainable ecosystem services, for example, 60 in nutrient depleted agricultural soils (Öpik *et al.*, 2006). AM fungi are close to being eradicated from agricultural land under current practices which include; tillage (Johansson *et al.*, 2004), crop rotation (Plenchette *et al.*, 2005) and application of agrochemicals (Bünemann *et al.*, 2006).

In recent years, the use of commercially available AM inocula to restore native AM fungal 65 communities has been investigated with the objective of maximising the many biotic and abiotic benefits of an intact symbiosis. These benefits include i) improved nutrient acquisition, ii) stabilisation of soil aggregates, iii) prevention of erosion and iv) alleviation of potential plant stress factors (Wang *et al.*, 2011, Andrade *et al.*, 2013). These investigations have often presented conflicting results (Gianinazzi & Vosátka, 2004, 70 Rowe *et al.*, 2007, Faye *et al.*, 2013). What these studies highlight is that optimisation of AM inoculation strategies requires specialist management practices. These practices include i) careful inoculum selection to ensure quality and suitability to the host plant, ii) minimal tillage, iii) intelligent crop rotation (non-mycorrhizal should not precede mycorrhizal crops), iv) organic fertilisation practices, and v) avoiding use of

75 agrochemicals i.e. pesticides (Dodd & Ruiz-Lozano, 2012, Berruti *et al.*, 2014) wherever possible.

Stricter governance of air pollution has led to greatly reduced inorganic S deposition to soils. A simultaneous increase in high yielding crop varieties has further depleted soil of plant available SO_4^{2-} stocks (McGrath *et al.*, 2003). Soil S exists up to 95% organically
80 bound (Autry & Fitzgerald, 1990), predominantly as aliphatic and aromatic sulfonates (30-70%) and sulfate esters (20-60%), and is not directly available to plants (Zhao *et al.*, 2006). Hydrolysis of the sulfate ester bond is facilitated by bacteria and saprotrophic fungi, however, aromatic sulfonate mobilisation is facilitated solely by specific bacteria (Schmalenberger & Kertesz, 2007). Many bacteria isolated from soil possess the ability
85 to mobilise aliphatic sulfonates (King & Quinn, 1997), however, aromatic sulfonate mobilisation has been shown to be more deterministic of plant S nutrition. Indeed, mobilisation of aromatic sulfonates has been shown to promote the growth of tomato plants (Kertesz & Mirleau, 2004) and *Arabidopsis* plants (Kertesz *et al.*, 2007). The genetic organisation of this activity is catalysed by a FMNH₂-dependent monooxygenase
90 enzyme complex encoded in the *ssu* gene cluster (Eichhorn *et al.*, 1999). The monooxygenase SsuD cleaves sulfonates to aldehydes and the reduced flavin for this process is provided by the FMN-NADPH reductase SsuE. For aromatic desulfonation an additional *asfRABC* gene cluster is required and the *asfA* gene is a marker for this activity (Vermeij *et al.*, 1999, Schmalenberger & Kertesz, 2007).

95 Studies on aromatic desulfonating bacterial phylogeny identified numerous Beta-Proteobacteria; *Variovorax*, *Polaromonas*, *Hydrogenophaga*, *Cupriavidus*, *Burkholderia* (now *Paraburkholderia*) and *Acidovorax*, the Actinobacteria; *Rhodococcus* and the Gamma-Proteobacteria; *Pseudomonas* (Schmalenberger & Kertesz, 2007, Schmalenberger *et al.*, 2008, Schmalenberger *et al.*, 2009, Fox *et al.*, 2014). Additionally,

100 *Stenotrophomonas* and *Williamsia* species have been isolated from AM hyphae (Gahan & Schmalenberger, 2015).

Plants rely on microbial populations for mobilisation of otherwise unavailable nutrients from soil. AM fungi have been shown to play an important role in mobilisation of P (Read & Perez-Moreno, 2003) and N (Hodge *et al.*, 2001), however, their role in mobilisation
105 of the essential macro-nutrient S remains unclear. AM fungi increase the nutritive absorptive surface area of their host plant via their extraradical hyphae (ERH) providing a niche for interacting with organo-S mobilising bacteria (Barea *et al.*, 2002). Additionally, AM symbiosis alters the chemical composition of their host plants exudates which may stimulate functional bacterial populations (Lynch & Whipps, 1990). Addition
110 of methyl-ester sulfonates (MES) to soil was shown to stimulate ERH growth putatively as a result of sulfonate mobilising bacterial metabolites (Vilarino *et al.*, 1997). Enhanced ERH growth may stimulate further bacterial proliferation in a potential positive feedback loop.

The hypothesis of this study was that AM inoculation may increase colonisation rates,
115 host plant biomass yield, and alter organo-S mobilising microbial communities. Effectively manipulated, inoculation with AM fungi has potential to enhance output from agricultural land. It is imperative that the land is sustainably managed with limited inorganic fertilisation input whilst utilising the beneficial properties of AM fungi to reduce agrochemical requirement. It is necessary to understand the full potential of AM
120 inoculation practices as demand from agricultural land increases and available stocks of essential macro-nutrients for fertilisation are depleted (Smit *et al.*, 2009).

2. Materials and Methods

2.1. Site description

The soil used to create the subsequent experiments was obtained from Teagasc, Johnstown Castle, Co. Wexford, Ireland (52°16'N, 6°30'W, 30 m above sea level). The soil type is a poorly drained gley soil (pH 6), organic matter (11%), and loamy topsoil (18% clay), classified as Mollic Histic Stagnosol (WRB 2006). The soil used has not received P or S fertiliser since 1968 and has not been ploughed since 1970 (P0-0, site 5A). Swards are mixtures of *L. perenne*, *D. glomerata*, and various meadow grass species (Tunney *et al.*, 2010).

2.1.1. Bi-compartmental Microcosms

Bi-compartmental microcosms were established using 120 g of a one part sand (Glenview Natural Stone, Ireland) to soil (Teagasc, Johnstown Castle) mixture. These systems were established to ascertain the bacterial and fungal community response to inoculation with AM fungi. The bi-compartmental systems were composed of 2 Plexiglas plates (12 x 12 cm) (Access Plastics, Ireland) encasing two 0.5 cm layers of soil separated by a 35 µm nylon mesh layer (Plastok Associates Ltd, Great Britain). Mesh of this size is sufficiently small to allow mycorrhizal cross colonisation while preventing root access. In the first compartment, *Lolium perenne*, *Agrostis stolonifera*, or, *Plantago lanceolata* (Emorsgate Seeds, UK) were planted (Figure 1a) and a second root free compartment (Figure 1b) was either; left un-inoculated as a control (C treatment), inoculated with 1.8 g of *Rhizophagus irregularis*, purchased as *Glomus intraradices* (Glomus treatment), or inoculated with a commercial mix of 6 different AM fungi including; *G. intraradices* (now *Rhizophagus irregularis*), *G. mosseae* (now *Funneliformis mosseae*), *G. etunicatum*, *G. hoi*, *G. constrictum*, and *G. claroidium* (Mixed treatment) (Symbivit, Symbion, Czech Republic).

The commercial AM mixed inoculum is supplied as dry granules containing 150 infective propagules (IP) per g and additives in this inoculum include; natural clay carriers, bio-actives supporting AM symbiosis (chitin, keratin, natural humates, seaweed extract, and ground minerals), and powered biodegradable gel. The Glomus treatment did not contain these additives and the substrate used in this instance was perlite. For all 3 plants, treatments were carried out in quadruplicate and the plant systems were grown in an A1000 Adaptis plant growth chamber (Convion, Germany) with day-night temperature of 25-15 °C, respectively, a 12 h day length, 70% relative humidity (RH), and 320 $\mu\text{mol m}^{-2}\text{s}^{-2}$ photosynthetically active radiation (PAR). The systems were watered and misted with distilled water (dH₂O) three times a week, and fortnightly supplemented with modified Hoagland's solution (50% nutrient concentration but without any S, S free) (Hoagland and Snyder 1933). After 6 months of growth, the systems were harvested and analysed for bacterial and fungal community diversity and bacterial sulfonate mobilising activity.

Figure 1 - Bi-compartmental microcosms (A) the plant root compartment (B) the root free – arbuscular mycorrhiza compartment, and a selectively penetrable nylon mesh (35 μm).

2.1.2. PGP pot experiments

Pot experiments were established using *A. stolonifera* as host plant to determine the impact of AM inoculation practices on PGP (Plant Growth Promotion) and on sulfonate mobilising bacterial communities. Plants of *A. stolonifera* were grown in 200 g of one part sand to soil mixture. Four experiments were established in replicates of 5 (Figure 2).

Figure 2 – Plant Growth Promotion pot experiments with *Agrostis stolonifera*. Control = free of inoculant, AC = autoclaved inoculum, *Glomus* = *Rhizophagus irregularis* inoculum, Mixed = AM mix inoculum, Bulk Soil = untreated soil.

The first control did not receive AM inoculant to assess the activity of the native AM fungal community and compare that with post-inoculation treatments. The second control received an inactivated commercial mixed inoculum (see above; autoclaved for 15 min, 121 °C) to establish if there was any abiotic element in the inoculum exerting a PGP effect (Autoclaved control = AC). The third treatment received a mono species *Rhizophagus irregularis* inoculation (*Glomus*), while the final treatment received the viable mixed AM fungi inoculation (Mixed). For all experiments, each pot received 100 *A. stolonifera* seeds and, for all experimental treatments (AC, *Glomus* and Mixed), 3 g of the respective

180 inoculant. All treatments were grown for 10 weeks in an A1000 Adaptis plant growth chamber (Conviron, Germany) as described above.

2.2. Percentage root colonisation

At the time of harvest for both bi-compartmental microcosms and PGP pot experiments, each plant and treatment was examined for percentage root colonisation by AM fungi
185 using a modified version of the grid line intersect technique (McGonigle *et al.*, 1990). A representative population of roots (2 root fragments, 10 cm in length were picked from the top, middle, and bottom of the microcosms/pots) and cut into 1 cm segments. The roots were stained with 10% KOH (w/v) for 12 h. The segments were washed with dH₂O and covered with alkaline H₂O₂ to bleach for 60 min. The bleaching solution was
190 discarded and the roots were rinsed thoroughly with water. Roots were acidified in a 0.1 M HCl solution for 12 h to ensure staining of intracellular fungal structures. The HCl solution was discarded and the roots were covered with a lactoglycerol trypan blue stain (lactic acid: glycerol: H₂O in a 1:1:1 ratio, with 0.05% (w/v) trypan blue) and incubated at 90 °C for 45 min. The stained roots were then removed and covered in lactoglycerol
195 de-stain (minus trypan blue) overnight prior to examination. The 1 cm root segments were examined one field of view at a time (x 1000 magnification). The field of view was moved in reference to a graticule inserted into the eyepiece and the point of intersection was determined at the position of the graticule's vertical crosshair intersecting the root (McGonigle *et al.*, 1990).

200 Once the point of intersection was noted, the field of view was moved across the surface area of the root and the presence of (1) arbuscules (2) vesicles and (3) hyphae was noted as 'negative' (no fungal structures), 'arbuscules', 'vesicles', or 'hyphae only'. If the crosshair intersected an arbuscule or vesicle, the respective category was increased by

one and the total number of intersections was also increased by one. This was also the
 205 case for the ‘hyphae only’ category. In the case of both arbuscules and vesicles being
 recorded at an intersection, the individual categories were both incremented but the total
 number of intersections was increased only by one. Arbuscular and vesicular colonisation
 (AC and VC, respectively) was calculated by dividing their respective counts by the total
 number of intersections examined i.e. if AC was observed in 50 of 100 intersections:
 210 $50/100 = 0.5$ or 50%. Hyphal colonisation (HC) was calculated as a proportion of the non-
 negative intersections. For both bi-compartmental microcosms and pot experiments, 3
 replicates of 100 intersections per treatment, per plant were examined to calculate AM
 fungal colonisation.

215 2.3. *Extraction and quantification of bacteria from AM hyphae*

2.3.1. *Bi-compartmental microcosms*

To harvest the bi-compartmental microcosms, in-depth morphological assessment was
 used to pick 0.5 g of hyphae from the root free compartment of the control experiment
 and the inoculation treatments using a compound microscope (x 100 magnification) and
 220 fine forceps (Hodge & Fitter, 2010). Detailed microscopic observations of the picked
 hyphae confirmed typical anatomical features of AM fungi and the absence of defined
 septa (Humphreys *et al.*, 2010). As a control, 0.5 g of bulk soil (BS), i.e. soil not under
 the influence of roots or hyphae, was analysed in parallel. Loosely attached soil was
 removed from the picked hyphae by dipping in sterile dH₂O, and associated bacteria were
 225 then extracted into 10 mL of sterile saline solution (0.85% w/v) by shaking at 75 rpm on
 an Elmi Intelli-Mixer RM-2 (Elmi Tech Ltd, Latvia) for 30 min at 4 °C. At this point, an

aliquot was taken and used to quantify bacterial communities. Following this, the treatments were centrifuged at 4500 rpm and 4 °C for 20 min and the pellet was frozen immediately (-18 °C) for downstream cultivation independent community analysis.

230 Using the aliquot of bacterial suspension, 100 µL was added to 900 µL of saline solution (0.85% w/v) and a series of 7 tenfold serial dilutions were performed. Most Probable Number (MPN) analysis was undertaken in liquid R2A (Reasoner & Geldreich, 1985) and MM2TS (Fox *et al.*, 2014) to enumerate the cultivable heterotrophic and sulfonate mobilising communities. A 20 µL aliquot from each dilution (10^1 - 10^7) was added to 200
235 µL of either R2A or MM2TS in 96 well microtitre plates. Following 2 weeks of growth in an Innova Incubator Shaker Series (New Brunswick Scientific, UK) at 75 rpm and 25 °C, the OD (590 nm) was recorded after 3 minutes shaking (Intensity Level 3) using an ELX808IU spectrophotometer (Bio Tek Instruments Inc., Winooski, VT). The OD₅₉₀ was used to identify the lowest dilution with growth in all five wells, the number of wells with
240 growth in the subsequent two dilutions was used to generate a three digit MPN number and this was used to refer to an MPN table to obtain an MPN g⁻¹ value (FDA, 2011). This MPN g⁻¹ value was substituted into the following equation: MPN g⁻¹ x (1/V) x (DF). V is the volume plated in mL and DF is the dilution factor (0.02 mL and 20, respectively for this experiment).

245 In order to ascertain the taxonomic diversity of sulfonate mobilisers, a 100 µL aliquot of bacterial suspension was removed from 3 wells of the liquid MM2TS for the control and each treatment and spread on solid MM2TS under aseptic conditions (Beil *et al.* 1995) using 6 g L⁻¹ of low-S agarose as solidifying agent. After one week, 20 single colonies were selected and sub-cultured for each plant type and treatment. DNA was extracted
250 from each isolate using the quick lysis protocol (Schmalenberger *et al.*, 2001) and 16S

rRNA gene amplification was undertaken with a final concentration per 25 µL reaction of 1 X buffer (2 mM MgCl₂), 0.2 mM dNTP mix, 0.4 µmol of each primer 27F and 1492R (Lane, 1991) (Table 1) and 0.5 U of DreamTaq polymerase (Fisher Scientific, Waltham, MA). All PCR reactions were carried out in a G-Storm GS2 thermocycler (G-Storm, UK).

255 The cycling conditions were as follows; initial denaturation of 95 °C for 5 min, 32 cycles of 94 °C denaturation (45 s), 55 °C annealing (45 s) and 72 °C extension (90 s). Final extension was undertaken at 72 °C for 5 min.

To identify dominant OTUs (Operational Taxonomical Units), RFLP (Restriction Fragment Length Polymorphism) was carried out on PCR amplicons using the restriction
260 enzymes *RsaI* and *TaqI* (5 U per reaction; Thermo Scientific) for 4.5 h at 37 °C. The digested DNA was run on a 2% agarose gel at 100 V for 40 min. Dominating OTUs were screened for presence of the sulfonate mobilising *asfA* gene using the functional *asfA* primers asfAF2 and asfBtoA (Schmalenberger and Kertesz 2007) (Table 1). The *asfA* gene was amplified using the following PCR protocol; initial denaturation of 94 °C for 4
265 min, 10 cycles of 94 °C denaturation (45 s), 67–57 °C touchdown (30 s), and extension of 72 °C (90 s), plus 25 further cycles at with an annealing temperature of 55 °C. The PCR was conducted using a Kapa 2G Robust PCR kit (Kapa Biosystems, Woburn, MA, USA) in 25 µL reactions containing; 1 x Buffer A, 1 x Enhancer, 5% DMSO (Sigma-Aldrich), 2 mM MgCl₂, 0.2 mM dNTP, 0.5 µM of each primer and 0.5 U of the Kapa
270 Robust polymerase.

Subsequently, amplified 16S and *asfA* genes of dominant OTUs were purified (GenElute, Sigma-Aldrich, St. Louis, MO) and quantified using a Nano Drop ND-1000 (Thermo Scientific, Waltman, MA, USA) before being subjected to Sanger sequencing (GATC Biotech, Germany) (Sanger and Coulson 1975).

275 2.3.2. PGP pot experiments

Following a 10-week growth period, the PGP pot experiments were harvested. An in-depth morphological assessment was used to pick 0.5 g of hyphae from the roots of *A. stolonifera* for the control, and the three inoculation treatments (AC, Glomus and Mixed) using a compound microscope (x 100 magnification) (Hodge & Fitter, 2010). Bacteria
280 were extracted and MPN analysis was undertaken in MM2TS media as described above. Additionally, at the time of harvest all above and below ground plant material was dried at 55 °C for 72 h in a fan assisted oven (Carbolite, UK) after which the dry weights were recorded. All excess soil was removed from the roots using a fine bristled brush before drying.

285 2.4. Community fingerprinting

Community fingerprinting analysis was undertaken for the bi-compartmental microcosm experiments. The frozen pellets of the bacterial extractions were used for community DNA extractions using the UltraClean Soil DNA extraction kit from MoBio (Carlsbad, CA). The extracted DNA was used for PCR-DGGE of bacterial 16S rRNA and Fungal
290 ITS communities for the bi-compartmental microcosms.

Bacterial 16S rRNA gene amplification was carried out using the primer pair GC-341F/518R (Table 1) targeting the V3 region for DGGE (Muyzer *et al.*, 1993). The final concentration in each 25 µL reaction was; 1 X buffer (2 mM MgCl₂), 0.2 mM dNTP mix, 0.4 µmol of each primer and 0.5 U of DreamTaq polymerase (Fisher Scientific, Waltham,
295 MA). A touchdown PCR protocol was used with the following cycling conditions; initial denaturation of 95 °C for 5 min, 20 cycles of 94 °C denaturation (45 s), 65-55 °C touchdown (45 s), 72 °C extension (45 s), plus 18 further cycles with an annealing

temperature of 55 °C. Final extension was undertaken at 72 °C for 5 min. DGGE was carried out on 200 x 200 x 1 mm gels in a TV400 DGGE apparatus (Scie-Plas, Cambridge, UK). Gels of 10% (w/v) acrylamide/bisacrylamide were prepared and run using a linear 30-60% gradient in 1 x TAE buffer (60 °C) for 16.5 h at 63 V (Fox *et al.*, 2014). After completion, gels were stained in SYBR Gold (1:10,000 diluted) (Invitrogen, Carlsbad, CA) for 30 min and the image taken on a Syngene G:Box (Cambridge, UK).

Fungal DNA fingerprinting was undertaken using the fungal specific primer ITS-1F (Gardes & Bruns, 1993) and ITS-4 (White *et al.*, 1990) (Table 1). This product was tenfold diluted and used as template in a nested PCR using the primer pair ITS-1FGC (Bougoure & Cairney, 2005) and ITS-2 (White *et al.*, 1990) (Table 6). Both PCRs were undertaken in 25 µL reactions each containing; 1 X buffer (2 mM MgCl₂), 1 M betaine, 0.2 mM dNTP mix, 0.4 µmol of each primer and 0.5 U of DreamTaq polymerase (Fisher Scientific, Waltham, MA). For both PCR reactions, amplification was performed as follows: initial denaturation of 95 °C for 5 min, 40 cycles at 95 °C denaturation (45 s), 60 °C annealing (45 s) and 72 °C extension (45 s), and 20 further cycles with an annealing temperature of 50 °C (Gardes & Bruns, 1993). Final extension was undertaken at 72 °C for 5 min. DGGE was run as before with a gradient of 35-65%.

315

2.5. Diversity of AM fungi and *AsfA*

AM and *asfA* gene diversity was analysed by generating a clone library of the Control, Glomus and Mixed treatment of *L. perenne*. For AM fungal diversity, the AM specific primer AM1 (Helgason *et al.*, 1998) alongside the universal eukaryotic primer NS31 (Simon *et al.*, 1992) (Table 1) were used to target the AM fungal 18S rRNA gene. The

320

final concentration per 25 µL PCR reaction was; 1 X buffer (2 mM MgCl₂), 1 M betaine, 0.2 mM dNTP mix, 0.4 µmol of each primer and 0.5 U of DreamTaq polymerase (Fermentas, Waltman, MA). The PCR was carried out under the following conditions; initial denaturation of 95 °C for 5 min, 30 cycles of 94 °C denaturation (30 s), 58 °C
325 annealing (60 s) and 72 °C extension (90 s). Final extension was undertaken at 72 °C for 5 min.

For diversity of sulfonate mobilising bacteria, the *asfA* gene was amplified with primers asfAF1all (Gahan & Schmalenberger, 2015) and asfBtoA (Schmalenberger & Kertesz, 2007) (Table 1). A touchdown PCR was carried out under the following conditions; initial
330 denaturation of 95 °C for 5 min, 10 cycles of 95 °C denaturation (30 s), 65-55 °C touchdown (30 s) and 68 °C extension (40 s), and 25 further cycles at 55 °C annealing. Final extension was undertaken at 68 °C for 5 min. PCR was formulated in 25 µL reactions each containing; 1 X Terra PCR Direct Buffer (2 mM MgCl₂), 0.2 mM dNTP mix, 0.4 µmol of each primer and 0.7 U of Terra PCR Direct Polymerase (ClonTech
335 Europe, Saint-Germain-en-Laye, France).

The respective PCR products were purified, quantified (see above) and ligated into the cloning vector pJET1.2/blunt (CloneJet, Thermo Scientific). The respective ligation reaction was transformed into *E. coli* DH5α. To ascertain taxonomic diversity of recombinant plasmids containing an insert of the correct size, RFLP was carried out on
340 PCR amplicons using 10U of the restriction enzymes *AluI* and *RsaI* (Thermo Scientific) in the provided buffer overnight. Clones with matching restriction patterns were classified as a single genotype (OTU) using Phoretix 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Unique genotypes with more than one representative clone were re-amplified and the purified PCR product was used for sequence identification (GATC

345 Biotech). The sequences obtained were subjected to gene comparison using BLAST
(Altschul *et al.*, 1990). Sequences of *asfA* were imported into arb (Version 5.2) (Ludwig
et al., 2004), translated into proteins and integrated into an *asfA* phylogenetic tree
(Schmalenberger *et al.*, 2010).

350

Table 1 – Primers used in this study

ID	Sequence	Target	Reference
27F	5'-AGAGTTTGATCMTGGCTCAG-3'	16S rRNA	Lane 1991
1492R	5'-GGTTACCTTGTTACGACTT-3'	16S rRNA	Lane 1991
341F-GC	5'-CGCCCGCCGCGCGCGGCGGGGCGGGGGCACGGGGGGCCTCGGGACC GAGCAG-3'	16S rRNA	Muyzer et al. 1993
518R	5'-ATTACCGCGGCTGCTGG-3'	16S rRNA	Muyzer et al. 1993
AsfAF2	5'-TACATGCGSCTGATGCGCA-3'	<i>asfA</i> gene	Schmalenberger and Kertesz 2007
AsfBtoA	5'-ASCTCGCACATGAAGCAGG-3'	<i>asfA</i> gene	Schmalenberger and Kertesz 2007
asfAF1all	5'-YTSTCVGGCATGGAGTTYT-3'	<i>asfA</i> gene	Gahan and Schmalenberger 2015
ITS-IF	5'-CTTGGTCTTTAGAGGAAGTAA-3'	ITS fungal	Gardes and Bruns 1993
ITS-4	5'-TCCTCCGCTTATTGATATGC-3'	ITS fungal	White et al. 1990
ITS-IFGC	5'-CGCCCGCCGCGCGCGGCGGGGCGGGGGCGCGGGGGCCTTGGTCTTTA GAGGAAGTAA-3'	ITS fungal	Bougoure and Cairney 2005
ITS2	5'-GCTGCGTTCTTCATCGATGC-3'	ITS fungal	White et al. 1990
AM1	5'-GTTTCCCGTAAGGCGCCGAA-3'	18S rRNA	Helgason et al. 1998)
NS-31	5'-TTGGAGGGCAAGTCTGGTGCC-3'	18S rRNA	Simon et al. 1992

353 2.7. Data analysis

354 Univariate analysis of MPN, root colonisation and PGP data was carried out using SPSS
 355 (Version 22.0; IBM, USA). DGGE fingerprinting gels were digitalised and band patterns
 356 analysed with the software package Phoretix 1D (Nonlinear Dynamics, UK). Cluster
 357 analysis via UPGMA was carried out and obtained band pattern matrices were exported
 358 for DCA and permutation tests (Monte-Carlo with 9,999 replicates) as described
 359 previously (Schmalenberger *et al.*, 2010).

360 **3. Results**

361 *3.1. PGP effect of inoculation with AM fungi*

362 Following a 10 week growth period in pots, there was an increase in the aboveground dry
 363 weight of plants inoculated with the Mixed, Glomus and Control-AC over the Control
 364 treatment ($P < 0.05$) (Figure 3). Additionally, Mixed treatment had increased
 365 aboveground dry weight over the Glomus and Control-AC ($P < 0.05$). There was not a
 366 difference between Glomus and Control-AC ($P > 0.05$). The increased aboveground
 367 biomass obtained with the Glomus treatment was attributed to the presence of the AM
 368 fungus, whereas, the Control-AC inoculum contained commercial additives that may
 369 have led to the increased biomass obtained over the control. Mixed treatment had the
 370 largest mean aboveground dry weight (1.24 ± 0.05 g) and the Control experiment had the
 371 lowest mean aboveground dry weight of (0.90 ± 0.06 g) (Figure 3A).

372 The belowground dry weight was analysed and increased root/hyphae biomass was
 373 observed for Mixed over the Glomus treatment and controls ($P < 0.05$) (Figure 3). There
 374 was not a difference in biomass for the Control, Control-AC or Glomus treatments ($P >$
 375 0.05). Mixed treatment had the largest mean belowground dry weight at 2.37 g, while the
 376 remaining experiments had a mean dry weight of 1.71 to 1.97 ± 0.18 g (Figure 3B).

377

378 **Figure 3A – Comparison of the dry weight of *Agrostis stolonifera* aboveground biomass. Control =**
379 **free of inoculant, Control AC = autoclaved inoculum, *Glomus* = *R. irregularis* inoculum, Mixed =**
380 **mixed inoculum. Letters (a-c) indicate significant differences.**
381

382

383 **Figure 3B – Comparison of the dry weight of *Agrostis stolonifera* belowground biomass. Control =**
384 **free of inoculant, Control-AC = autoclaved inoculum, *Glomus* = *R. irregularis* inoculum, Mixed =**
385 **mixed inoculum. Letters (a-b) indicate significant differences.**
386
387

388 3.2. AM colonisation

389 3.2.1 Bi-compartmental microcosms

390 For all three plants, intracellular AC and VC increased following both the *Glomus* and
 391 Mixed treatment over the C treatment ($P < 0.05$) (Figure 4). Additionally, there was an
 392 increase in AC following Mixed over *Glomus* treatment for *L. perenne* and *A. stolonifera*
 393 ($P < 0.05$) (Figure 5A, B), this effect was not observed for *P. lanceolata* ($P > 0.05$) (Figure
 394 4C). There was not an additional increase in VC with Mixed over *Glomus* treatment for
 395 the three plants analysed ($P > 0.05$). Finally, there was not an effect of AM inoculation
 396 on HC ($P > 0.05$) due to the presence of dense networks of hyphal mycelia surrounding
 397 the plant root which made it difficult to identify points of infection (Figure 4).

Figure 4 – Arbuscular mycorrhizal root colonisation for *Lolium perenne* (A), *Agrostis stolonifera* (B) and *Plantago lanceolata* (C). Arbuscular colonisation = AC, vesicular colonisation = VC, and hyphal colonisation = HC. Treatments include Control = C, Glomus = G (*R. irregularis* inoculum), and Mixed = M (6 AMF species inoculum). Letters (A-B, a-b, γ) indicate significant differences.

3.2.2 PGP pot experiments

The PGP pot experiments demonstrated similar colonisation rates and sensitivity to AM species as was observed with the bi-compartmental microcosms. Arbuscular colonisation was increased following both *Glomus* and Mixed inoculation over the Control and the Control-AC treatments ($P < 0.05$). There was not an increase in colonisation rate with Mixed over *Glomus* inoculations or Control-AC over the un-inoculated Control ($P > 0.05$) (Figure 5).

Colonisation rates were 16% and 20% for control and Control-AC whereas for *Glomus* and Mixed treatment the colonisation rate was 68% and 80%, respectively (Figure 5). This result indicates that inoculation with active AM inocula increased colonisation capacity.

417

Figure 5 – Arbuscular mycorrhizal root colonisation for Plant Growth Promotion (PGP) pot experiments with *Agrostis stolonifera*. Control = free of inoculant, Control-AC = autoclaved inoculum, *Glomus* = *R. irregularis* inoculum, Mixed = AM mix inoculum. Letters (A-B) indicate significant differences.

422

423 3.3. Quantification of cultivable heterotrophic microbes

424 3.3.1 Bi-compartmental microcosms

425 For all plants, more abundant bacterial communities were observed in R2A and MM2TS
 426 media for picked hyphae (Control, Glomus, and Mixed) over untreated bulk soil ($P <$
 427 0.01) (Figure 6). Additionally, higher abundances were observed with Mixed and Glomus
 428 treatment over the Control experiment for *L. perenne* and *P. lanceolata*, respectively (P
 429 < 0.05) (Figure 6A, C). However, an additional increase in abundance of either the total
 430 cultivable heterotrophic or the sulfonate mobilising bacterial community was not
 431 observed between Mixed and Glomus treatment ($P > 0.05$) (Figure 6A, C). For *A.*
 432 *stolonifera*, there was not an increase in abundance of either the total cultivable
 433 heterotrophic or the sulfonate mobilising bacterial community with Mixed or Glomus
 434 treatment over the Control experiment ($P > 0.05$) (Figure 6B, C). However, the total
 435 cultivable heterotrophic and the sulfonate mobilising bacterial communities were more
 436 abundant for picked hyphae and bulk soil for *A. stolonifera* and *P. lanceolata* over *L.*
 437 *perenne* (Figure 6). For *L. perenne*, growth from bulk soil was below the detection limit
 438 in MM2TS (1000 MPN g⁻¹) (Figure 6A), while for *A. stolonifera* and *P. lanceolata*, it
 439 ranged from 10³ - 10⁴ MPN g⁻¹ suggesting a higher indigenous community in these plant
 440 microcosms (Figure 6B, C).

Figure 6 – Most probable number (MPN) analysis of bacteria in microcosms with *Lolium perenne* (A), *Agrostis stolonifera* (B) and *Plantago lanceolata* (C). R2A and MM2TS were used to quantify the total cultivable bacterial community and sulfonate mobilisers, respectively. Control = free of inoculant, Glomus = *R. irregularis* inoculum, Mixed = AM mix inoculum, Bulk Soil = untreated soil. Letters (A-B-C, a-b-c) indicate significant differences. Below detection limit = BDL.

3.3.2. PGP pot experiments

For the PGP pot experiment, as observed for the microcosms, the sulfonate mobilising bacterial community abundance was higher for the picked hyphae over the bulk soil ($P < 0.05$) (Figure 7). For the pot experiments unlike the microcosms, a higher abundance of sulfonate mobilisers were observed for both Glomus and Mixed treatments over both the Control and the Control-AC (autoclaved inoculum) treatment ($P \leq 0.05$) (Figure 7). A difference was not observed between both controls, or between both AMF treatments ($P > 0.05$). Differences between the results obtained for the pot experiments and bi-compartmental microcosms can be attributed to their set-up (pot versus microcosm) and growth period (10 weeks versus 6 months).

460

461

462 **Figure 7 – Most probable number (MPN) analysis of the bacterial community in the plant growth**
 463 **promotion pot experiment with *Agrostis stolonifera*. MM2TS were used to quantify the cultivable**
 464 **sulfonate mobilising community. Control = free of inoculant, Control-AC = autoclaved inoculum,**
 465 **Glomus = *R. irregularis* inoculum and Mixed = AM mix inoculum, Bulk Soil = untreated soil. Letters**
 466 **(A-C) indicate significant differences.**

467 3.4. Screening of taxonomic diversity of cultivable sulfonate mobilisers

468 For the 60 isolates taken from the Control, 9 OTUs were obtained in total (based on 16S
469 rRNA gene typing). Of these, 3 were found in *L. perenne*, and 5 in *A. stolonifera* and *P.*
470 *lanceolata* respectively. Two of these OTUs (OTUs 1 and OTU3) were discovered in all
471 three plants and were present in both the Control and the AMF treatments (Table 2),
472 suggesting that they are common hyphosphere sulfonate mobilisers. The remaining OTUs
473 (OTUs 2, 11, 12, 13, 23, 24, and 25) were present only in the Control experiment and
474 only associated with one of the three plants (Table 2). This result suggests that inoculated
475 soil harbours a distinct community of sulfonate mobilising bacteria.

476 For the 60 isolates taken from the Glomus treatment, 8 OTUs were obtained. Of these, 4
477 were recovered from *L. perenne* (OTUs 4, 5, 6, and 7), 6 from *A. stolonifera* (OTUs 1, 4,
478 6, 15, 26 and 27), and 5 from *P. lanceolata* (OTUs 1, 4, 5, 6, and 15), respectively. The
479 OTUs 7, 26 and 27 were specific to one of the three plants and the Glomus treatment
480 (Table 2). The remainder of the OTUs overlapped across experiments and plants;
481 however, OTUs 4, 5, 6, and 15 were only present following inoculation in the Glomus or
482 Mixed treatments (Table 2).

483 For the Mixed treatment, 19 OTUs were obtained. Of these, 6 were obtained from *L.*
484 *perenne*, and 7 and 12 from *A. stolonifera* and *P. lanceolata*, respectively. The OTUs 1,
485 3, 4, 5, 6 and 15 were present for more than one plant and more than one experiment
486 (Table 2). There was minimal overlap of OTUs across Control, Glomus and Mixed
487 experiments suggesting that each treatment harbours a distinct cultivable sulfonate
488 mobilising community (Table 2). OTUs 1 and 3 overlapped with the Control experiment,
489 while OTUs 4, 5, 6, and 15 overlapped with the Glomus treatment. The remaining 13
490 OTUs were exclusive to the Mixed treatment (OTUs 8, 9, 10, 14, 16, 17, 18, 19, 20, 21,

491 22, 28, and 29). Additionally, the Mixed treatment had a higher number of OTUs than
 492 Control and Glomus experiments combined (19 compared to 9 and 8 for Control and
 493 Glomus, respectively) suggestive of a higher diversity of sulfonate mobilisers following
 494 this inoculation treatment.

495

496 **Table 2 – Dominant Operational Taxonomic Units (OTUs) of bacteria extracted from the AM hyphae**
 497 **of *Agrostis stolonifera* (*Agrostis*), *Lolium perenne* (*Lolium*), and *Plantago lanceolata* (*Plantago*) either**
 498 **un-inoculated (Control), inoculated with *R. irregularis* (Glomus), or inoculated with a mix of 6**
 499 **arbuscular mycorrhizal fungi (Mixed). Symbols mean OTU occurred in either * = all plants, † = all**
 500 **treatments, α = Control, β = Glomus, or γ = Mixed.**

OTU ID	Isolation Source			Occurrence per Treatment (%)			Community (%)
	<i>Lolium</i>	<i>Agrostis</i>	<i>Plantago</i>	Control	Glomus	Mixed	
1*†	✓	✓	✓	55	12	17	25
2 α	✓			8			3
3* $\alpha\gamma$	✓	✓	✓	23		7	9
4* $\beta\gamma$	✓	✓	✓		30	10	13
5* $\beta\gamma$	✓	✓	✓		15	20	12
6* $\beta\gamma$	✓	✓	✓		23	3	9
7 β	✓				7		2
8 γ	✓					12	4
9 γ	✓					7	2
10 γ	✓					3	1
11 α			✓	3			1
12 α			✓	3			1
13 α			✓	2			1
14 β			✓			5	1.5
15 $\beta\gamma$		✓	✓		5	7	3.5
16 γ			✓			2	1
17 γ			✓			2	1
18 γ			✓			2	1
19 γ			✓			2	1
20 γ			✓			2	1
21 γ			✓			2	1
22 γ			✓			2	1
23 α		✓		5			1.5
24 α		✓		3			1
25 α		✓		3			1
26 β		✓			5		1.5
27 β		✓			5		1.5
28 γ		✓				2	1
29 γ		✓				2	1

501 3.5. Functionality of dominant OTUs

502 In order to ascertain the sulfonate mobilising functionality of dominant OTUs (see 3.4
503 above), screening for the presence of the desulfonating *asfA* gene was undertaken. Of 180
504 bacterial isolates, 29 different restriction patterns were obtained (Table 2), of these, 9
505 OTUs were cultured from the control (C) experiment, 8 from the Glomus (G) treatment
506 and 19 from mixed (M) treatment. Of the 9 control experiment OTUs, 2 overlapped with
507 Glomus and/or Mixed treatment and 7 OTUs were exclusive to the Control experiment.
508 Of the 8 Glomus treatment OTUs, 5 overlapped with Control and/or Mixed experiment
509 and 3 OTUs were exclusive to the Glomus treatment. Of the 19 Mixed treatment OTUs,
510 6 overlapped with Control and/or Glomus experiment and 13 OTUs were exclusive to
511 Mixed treatment.

512 The dominant OTUs were then subjected to sequence identification and classes such as
513 *Alphaproteobacteria*, *Betaproteobacteria*, and the *Actinobacteria* predominated (Table
514 3). *Variovorax* was the dominating OTU at 25% of the sulfonate mobilising community
515 and was isolated in the hyphosphere of the Control and the AMF treatments (Table 3).
516 *Paraburkholderia* (13%), *Agrobacterium* (12%), *Polaromonas* (9%), *Rhodococcus* (9%),
517 and *Mesorhizobium* (4%) were also prevalent desulfonating bacteria. Interestingly, while
518 *Polaromonas* (9%) and *Rhodococcus* (9%) have previously been shown to have
519 desulfonating ability, *Paraburkholderia* (13%), *Agrobacterium* (12%), and
520 *Mesorhizobium* (4%) are newly associated with this capability. Furthermore, they were
521 only isolated from Glomus and/or Mixed microcosms (Table 3). All but OTU 6 and 8
522 (*Rhodococcus* and *Mesorhizobium*) were able to produce an amplification product in the
523 *asfA* PCR (see above).

524

525 Table 3 – Dominant desulfonating Operational Taxonomic Units (OTUs) obtained from the AM hyphae of *Agrostis stolonifera* (Agrostis), *Lolium perenne* (Lolium),
526 and *Plantago lanceolata* (Plantago) either un-inoculated (Control), inoculated with *R. irregularis* (Glomus), or inoculated with a mix of 6 arbuscular mycorrhizal
527 fungi (Mixed). Isolates were screened for amplification of *asfA* PCR product (+/-)

Class affiliation	Genus	OTU ID	Isolate ID	Closest Relative	Similarity %	Treatment	Plant	Total <i>asfA</i> %	
<i>Betaproteobacteria</i>	<i>Variovorax</i>	OTU1	Ag-C13	<i>V. paradoxus</i>	100	Control, Glomus, Mixed	All	25	+
<i>Betaproteobacteria</i>	<i>Paraburkholderia</i>	OTU4	Pl-M10	<i>P. bryophila</i>	99	Glomus, Mixed	All	13	+
<i>Alphaproteobacteria</i>	<i>Agrobacterium</i>	OTU5	Ag-G5	<i>A. rhizogenes</i>	99	Glomus, Mixed	<i>Lolium</i> , <i>Agrostis</i>	12	+
<i>Betaproteobacteria</i>	<i>Polaromonas</i>	OTU3	Ag-C37	<i>P. rhizosphaerae</i>	99	Control, Mixed	All	9	+
<i>Actinobacteria</i>	<i>Rhodococcus</i>	OTU6	Pl-C1	<i>R. erythropolis</i>	100	Glomus, Mixed	<i>Plantago</i> , <i>Agrostis</i>	9	-
<i>Alphaproteobacteria</i>	<i>Mesorhizobium</i>	OTU8	Lol-M17	<i>M. amorphae</i>	100	Mixed	<i>Lolium</i>	4	-

528

529 3.6. Community fingerprinting

530 3.6.1. 16S rRNA community diversity

531 Community fingerprinting for *L. perenne* (Figure 8A) illustrated that there was a bacterial
532 community shift following both AMF inoculations (Glomus and Mixed) over the Control
533 experiment ($P < 0.05$). Additionally, there was a community shift following Mixed over
534 Glomus treatment ($P < 0.05$). The results show that the bacterial community of *L. perenne*
535 is sensitive to AMF symbiosis and shifts in response to both the Glomus and Mixed
536 treatment.

537 The bacterial community response of *A. stolonifera* (Figure 8B) illustrated a shift
538 following Mixed over Control experiment ($P < 0.05$). However, there was not a
539 community shift following Glomus over Control or Mixed over Glomus treatment ($P >$
540 0.05). The results show that the bacterial community of *A. stolonifera* is sensitive to AMF
541 symbiosis; however, this response is dependent on the species of AM fungi used for
542 inoculation.

543 The bacterial community of *P. lanceolata* (Figure 8C) demonstrated a community shift
544 to both AMF inoculations over the Control experiment ($P < 0.05$). However, there was
545 not an additional shift following Mixed over Glomus treatment ($P > 0.05$). The results
546 obtained for all three plants demonstrate a bacterial community response to AMF
547 inoculation; however, this response is dependent both on the species of AM fungi used to
548 inoculate and the suitability of the plant-AM fungal partnership.

Figure 8 – Detrended correspondence analysis (DCA) bi-plot of the 16S bacterial rRNA community fingerprint for *Lolium perenne* (A), *Agrostis stolonifera* (B) and *Plantago lanceolata* (C). DCA1 = Axis 1, DCA2 = Axis 2. Treatments; Control = Triangle, Glomus = Square, Mixed = Diamond. Letters (A-B-C) indicate significant differences.

556 3.6.2. ITS fungal community diversity

557 Fungal community fingerprinting for *L. perenne* (Figure 9A) illustrated that there was a
 558 community shift following inoculation with Glomus treatment over the Control
 559 experiment ($P < 0.05$). However, there was not a fungal community shift following Mixed
 560 over either Glomus treatment or the Control experiment ($P > 0.05$). Fungal community
 561 fingerprinting for *A. stolonifera* and *P. lanceolata* (Figure 9B, C) illustrated that there
 562 was a community shift following both AMF treatments over the Control experiment ($P <$
 563 0.05). However, there was not a community shift following Mixed over Glomus treatment
 564 ($P > 0.05$).

565 These results demonstrate that the fungal community in the hyphosphere shifts in
 566 response to inoculation with AM fungi but that the response is not necessarily specific to
 567 the species of AM fungi present i.e. in the mono species or six species inoculation. As the
 568 current fingerprint approach includes both saprotrophic and mycorrhizal fungal species,
 569 the presence of AMF bands may give rise to differences in the fungal community
 570 composition present for the inoculation (Glomus and Mixed) treatments over the Control
 571 experiment.

Figure 9 – Detrended correspondence analysis (DCA) bi-plot of the fungal ITS region community fingerprint of *Lolium perenne* (A), *Agrostis stolonifera* (B) and *Plantago lanceolata* (C). DCA1 = Axis 1, DCA2 = Axis 2. Treatments; Control = Triangle, Glomus = Square, Mixed = Diamond. Letters (A-B) indicate significant differences.

3.7. Diversity of AM fungi and AsfA

3.7.1. Diversity of AM fungi

Clone libraries of AM fungal amplicons from the Glomus and Mixed treatments and the Control experiment of *L. perenne* were screened for different OTUs via RFLP. Screening of 150 clones (50 for each treatment) revealed 6 distinct OTUs with coverage of 100% for each library (Schmalenberger *et al.*, 2007). There were no overlapping genotypes/OTUs for the respective libraries. Upon BLAST analysis of dominant OTUs, all were found to be affiliated with members of the Glomeromycota. Thus validating the presence of AM fungi and demonstrating differences across treatments (Table 4). Due to the obligately bi-trophic nature of AM fungi there are limitations in AM sequence availability in reference databases that make taxonomic classification to genus and species level challenging.

Table 4 – Taxonomic assignment of representative AM fungal Operational Taxonomic Units (OTUs) derived from clonal AM sequence analysis from Control (C), *Glomus* (G) and Mixed (M) treatments of *Lolium perenne*.

ID	Affiliation	Rep. per OTU	Similarity [%]	Treatment
C1	<i>Acaulospora clone</i>	38	99	Control
C48	<i>Acaulospora clone</i>	12	99	Control
G1	<i>Uncultured mycorrhizal fungus</i>	38	100	Glomus
G32	<i>Acaulospora clone</i>	12	99	Glomus
M1	<i>Acaulospora clone</i>	25	99	Mixed
M7	<i>Scutellospora clone</i>	25	99	Mixed

594

595 3.7.2. Diversity of the sulfonate mobilising *asfA* gene

596 Clone libraries of the sulfonate mobilising *asfA* gene amplicons from Control, Glomus
597 and Mixed treatments of *L. perenne* were screened for diversity via RFLP. Screening of
598 150 clones (50 for each treatment) revealed 39 OTUs in total; 17 for the Control (3
599 overlapping), 18 for Glomus (6 overlapping), and 13 for Mixed treatment (8 overlapping).
600 Library coverage was 94%, 84% and 96% for each respective treatment (Schmalenberger
601 *et al.*, 2007). Of these 39 OTUs, 24 OTUs with more than two representatives were
602 subjected to DNA based sequence identification using BLAST, imported into arb (version
603 5.2), translated into proteins and incorporated into a phylogenetic tree (Figure 10 and 11).

604 There was a 12% overlap of Control treatment OTUs with Glomus and Mixed treatments
605 and only 3 OTUs occurred in both Control and either AM inoculation treatment (C15A,
606 C18A and C27B) (Table 5). There was a 36% overlap of Glomus and Mixed treatments
607 with 5 overlapping OTUs that dominate the post-inoculation sulfonate mobilising
608 community (G15A, G34A, G42A, M44A, and M45A) (Table 5). The results suggest that
609 the sulfonate mobilising bacterial community composition (*asfA* gene) shifts following
610 inoculation.

611 The sequences obtained were subjected to gene comparison using BLAST (Altschul *et*
612 *al.*, 1990). Sequences of *asfA* were imported into arb, translated into proteins and
613 integrated into an *asfA* phylogenetic tree (Schmalenberger *et al.*, 2010) using the
614 randomised accelerated maximum likelihood method. The majority of the Control clones
615 associated closely with the *Variovorax* and *Arthrobacter* clade while the Glomus and
616 Mixed treatment clones were most closely associated to *Paraburkholderia* (for continuity

in tree identified as *Burkholderia*), *Arthrobacter* and *Rhodococcus* clades (Figure 10, 12, respectively). Once again, suggesting that the sulfonate mobilising bacterial community composition is sensitive to inoculation with AM fungi.

Table 5 – Taxonomic assignment of representative desulfonating OTUs derived from clonal asfA DNA sequence analysis from AM hyphae of *Lolium perenne*, either uninoculated (Control), inoculated with *R. irregularis* (Glomus), or inoculated with a mix of 6 arbuscular mycorrhizal fungi (Mixed).

ID	Affiliation	Similarity [%]	Representatives per OTU/Treatment		
			Control	Glomus	Mixed
C13B	<i>Variovorax</i> species	80	3		
C15A	<i>Mycobacterium smegmatis</i>	79	6		2
C18A	<i>Mycobacterium smegmatis</i>	78	5		2
C21A	<i>Variovorax paradoxus</i>	86	4		
C25A	<i>Variovorax paradoxus</i>	97	2		
C27B	<i>Variovorax paradoxus</i>	82	2	1	
C30A	<i>Variovorax</i> species	81	2		
C31A	<i>Mycobacterium smegmatis</i>	79	3		
C41A	<i>Variovorax</i> species	80	3		
C42B	<i>Variovorax</i> species	82	3		
C43A	<i>Variovorax paradoxus</i>	81	4		
C43B	<i>Variovorax paradoxus</i>	86	4		
C4A	<i>Variovorax paradoxus</i>	86	4		
G15A	<i>Rhodococcus erythropolis</i>	97		6	5
G34A	uncultured <i>proteobacterium partial</i>	76		11	22
G38A	<i>Mycobacterium smegmatis</i>	79		8	
G42A	<i>Mycobacterium smegmatis</i>	74		1	2
G42B	uncultured <i>proteobacterium</i>	79		3	
M15B	uncultured <i>proteobacterium</i>	76			2
M29A	uncultured <i>proteobacterium partial</i>	76			3
M37A	uncultured <i>proteobacterium partial</i>	77			4
M44A	Uncultured <i>Variovorax</i> <i>sp.</i>	76		2	1
M45A	<i>Variovorax paradoxus</i>	86		2	3

624	M49A <i>uncultured</i> <i>proteobacterium</i>	72	4
-----	--	----	---

Figure 10 – Randomised accelerated maximum likelihood tree of truncated AsfA sequences with the Variovorax clade expanded. C, G and M indicate Control, Glomus and Mixed treatments. Cultivated (*italised*) and molecular isolates from this study are underlined. Molecular isolates from spring barley rhizospheres (sb; (Schmalenberger & Kertesz, 2007)), *Agrostis* grassland rhizospheres (CA; (Schmalenberger *et al.*, 2010)), wheat rhizospheres from Broadbalk (W; (Schmalenberger *et al.*, 2008)), rhizospheres and soils from the Damma glacier forefield (D; DA; (Schmalenberger *et al.*, 2010)) were isolated previously. Highlighted in red are clades to be expanded in Figure 11 below.

Figure 11 - Randomised accelerated maximum likelihood tree of truncated AsfA sequences with the clades minimised in the above figure (red) expanded. C, G and M indicate Control, Glomus and Mixed treatments. Cultivated (*italised*) and molecular isolates from this study are underlined. Molecular isolates from spring barley rhizospheres (sb; (Schmalenberger & Kertesz, 2007)), *Agrostis* grassland rhizospheres (CA; (Schmalenberger *et al.*, 2010)), wheat rhizospheres from Broadbalk (W; (Schmalenberger *et al.*, 2008)), rhizospheres and soils from the Damma glacier forefield (D; DA; (Schmalenberger & Noll, 2010)) were isolated previously. Highlighted in red are clades expanded in Figure 10 above.

4. Discussion

640 Numerous studies have shown that AM inoculation promotes plant growth (Wang *et al.*, 2011, Pellegrino *et al.*, 2012, Faye *et al.*, 2013). Indeed, AM fungi interact synergistically with functional soil microbial populations for P and N mobilisation (Hodge *et al.*, 2001, Read & Perez-Moreno, 2003, Hodge & Fitter, 2010). However, rarely has the effect of AM fungal inoculation and AM fungal associated microbiota been examined in the context of organo-S

645 mobilisation. This study aimed to investigate the impact of AM fungal inoculation practices on organo-S transforming microbial community dynamics to potentially improve nutrient supply and promote plant growth. The experiments undertaken demonstrated that AM inoculation significantly increases root colonisation, plant growth, and impacts organo-S mobilising microbial communities.

650 For the PGP pot experiments, the mono-species *R. irregularis* (Glomus) and mixed AM fungal treatments increased above and belowground plant biomass over the control experiment. Additionally, increases in biomass yield was less pronounced with the mono-species in comparison to the mixed inoculation, this result suggests that mixed AM inoculants have an increased probability of successfully forming an efficiently functional symbiosis. Indeed, many

655 studies corroborate this finding and show that mixed species inoculants exert more beneficial effects in comparison to their mono-species counterparts as a result of increased symbiotic potential arising from multiple species with differential characteristics (Rowe *et al.*, 2007, Faye *et al.*, 2013, Jin *et al.*, 2013). The autoclaved mixed inoculant increased growth more so than the un-inoculated control, however, the PGP effect was significantly less than that observed

660 with the biotic mixed inoculant. Rowe *et al.* (2007) demonstrated that increased growth can be a result of the substrate in which the inoculant is supplied. Indeed, in an experiment comparing commercial inoculants with 7 different host plants, 5 exhibited increased growth as a result of

the inoculant substrate alone thus providing an explanation for the PGP effect obtained with the present AC-Control inoculum (Rowe *et al.*, 2007).

AM inoculation significantly increased root colonisation for both the bi-compartmental microcosms and the PGP pot experiments. Tresender (2013) demonstrated a proportionality between increased AM root colonisation and both plant yield and nutrient content (Treseder, 2013), a potential mechanism for which is the increased efficiency of nutrient transfer via characteristic mycorrhizal structures (Van Der Heijden *et al.*, 1988, Klironomos & Hart, 2002).

An experiment carried out to ascertain the impact of commercial AM fungal inoculant on colonisation rates in *Pisum sativum* demonstrated that mono-species (*G. intraradices*) and mixed AM inoculant (*G. intraradices*, *G. mosseae*, and *G. clarum*) both significantly increased AM colonisation but there was not an additional benefit of mixed over mono-species inoculation (Jin *et al.*, 2013). Despite the equal colonisation capacity of both AMF treatments in the present study, the multi-species (Mixed) treatment increased biomass to a greater extent than the single species (Glomus) treatment. This may be the result of differential nutrient uptake efficiency for multiple distinct AM fungal species in the mixed treatment that is not associated with colonisation capacities and this leading to increased plant biomass yield (Marschner & Dell, 1994).

Cultivable community quantification revealed that total heterotrophic and sulfonate mobilising bacterial communities are larger in the hyphosphere than bulk soil and these findings are consistent with previous studies (Johansson *et al.*, 2004, Gahan & Schmalenberger, 2015). For the bi-compartmental microcosms, cultivable heterotrophic and sulfonate mobilising bacterial communities increased in abundance following Mixed treatment for *L. perenne*, only. For *P. lanceolata*, an increased cultivable heterotrophic community was observed in the *R. irregularis* single species treatment (Glomus). There was not an effect of AM inoculation on the sulfonate

mobilising community for *P. lanceolata* or *A. stolonifera*. However, the sulfonate mobilising communities were an order of magnitude higher for both plants. The chemical composition of root exudates is dependent on plant species and has been shown to alter microbial community composition and abundance (Lynch & Whipps, 1990, Grayston *et al.*, 1998). The characteristic root exudates of *A. stolonifera* and *P. lanceolata* may, therefore, have selectively stimulated larger indigenous communities of sulfonate mobilisers negating the requirement to expend the Control to AM fungi to further stimulate these populations (Jones *et al.*, 2004b). For the PGP pot experiment, unlike the bi-compartmental microcosms, increased abundances of sulfonate mobilisers were observed following both Glomus and Mixed inoculations. The duration of this experiment was 10 weeks in comparison to 6 months for the bi-compartmental microcosms. Evidence suggests that S requirement is highest at early stages of vegetative growth (Kertesz *et al.*, 2007, Gahan *et al.*, 2021), therefore, unlike the bi-compartmental microcosms, the Control may have been allocated to AMF associated microbial populations to facilitate mobilisation of sulfonates to fulfil the plants S requirement.

Bacterial isolates capable of mobilising sulfonates in possession of the *asfA* gene were found to belong to classes such as Alphaproteobacteria, Betaproteobacteria, and the Actinobacteria. Sequences identified include; *Variovorax*, *Paraburkholderia*, *Agrobacterium*, *Polaromonas*, *Rhodococcus*, and *Mesorhizobium*. *Variovorax*, *Polaromonas* and *Rhodococcus* species have previously been extracted from the rhizosphere of wheat and have a sulfonate mobilising *asfA* gene (Kertesz *et al.*, 2007, Schmalenberger & Kertesz, 2007, Schmalenberger *et al.*, 2008, Schmalenberger *et al.*, 2009). *Paraburkholderia*, *Agrobacterium* and *Mesorhizobium* species are newly associated with sulfonate mobilising activity and, furthermore, were only isolated from the AM inoculated treatments due to potential AM specific induced modification of the soil microbial environment (Badri *et al.*, 2013). The genera *Paraburkholderia*/*Burkholderia* and *Agrobacterium* have been linked with AM and bacterial interactions and PGP stemming

from their ability to mobilise P (Gentili & Jumpponen, 2006). Additionally, *Mesorhizobium* species have been shown to be involved in N fixation (Kaneko *et al.*, 2000) and PGP for both chickpea and barley by certain phosphate solubilising strains (Peix *et al.*, 2001). Cultivation independent analysis of the sulfonate mobilising *asfA* gene corroborates the cultivation dependent work and revealed minimal overlap of genotypes across treatments. The majority of the Control clones associated closely with the *Variovorax* and *Arthrobacter* clade while the Glomus and Mixed treatment clones were most closely associated to *Paraburkholderia*, *Arthrobacter* and *Rhodococcus* clades. This result demonstrates that AM inoculation stimulates shifts in the sulfonate mobilising bacterial community. Indeed, AM induced alteration of the sulfonate mobilising bacterial community has been observed previously with DNA extracted from hyphosphere of grassland swards (Gahan & Schmalenberger, 2015).

Community fingerprinting, undertaken for the bi-compartmental microcosms, identified a bacterial and fungal community shift following AMF inoculation. This was expected, as AM fungal symbiosis has been shown to alter microbial community composition in the hyphosphere (Johansson *et al.*, 2004, Gahan & Schmalenberger, 2015). Van der Heijden *et al.*, (1998) demonstrated using mono and mixed species inoculations, that growth promotion associated with AMF inoculation was only as good as the most effective AM species in the mix inoculated in isolation. This may provide an explanation for community shifts following the Glomus inoculation but no additional additive shift following Mixed inoculation as observed for *A. stolonifera* and *P. lanceolata*. As observed for root colonisation and cultivation-based analysis of desulfonating communities, *L. perenne* displayed the greatest sensitivity to both AMF inoculation treatments for the cultivation independent community fingerprinting. Helgason *et al.* (2002) provided an explanation for this by demonstrating that root colonisation, plant-fungal symbiont compatibility and subsequent overall plant vigour were subject to variation depending on the specific AM-plant fungal combination (Helgason *et*

al., 2002). It is important, therefore, to select a suitable AMF-plant symbiotic partnership before implementing AMF inoculation practices.

Inoculation with AM fungi can both promote plant growth and stimulate proliferation of bacteria involved in mineralisation of sulfonate-S. Interactions between AM fungi and bacteria have great potential for use in agriculture to improve S supply to plants when available SO_4^{2-} becomes a limiting factor to growth. The present study also highlights the selective nature of plant receptivity to AM fungi and the importance of careful selection of a high quality AM fungal inoculant suited specifically to the intended ecosystem.

5. Bibliography

- Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.
- Andrade MMM, Stamford NP, Santos CERS, Freitas ADS, Sousa CA & Junior MAL (2013) Effects of biofertilizer with diazotrophic bacteria and mycorrhizal fungi in soil attribute, cowpea nodulation yield and nutrient uptake in field conditions. *Scientia Horticulturae* **162**: 374-379.
- Autry AR & Fitzgerald JW (1990) Sulfonate S: a major form of forest soil organic sulfur. *Biology and Fertility of Soils* **10**: 50-56.
- Badri DV, Chaparro JM, Zhang R, Shen Q & Vivanco JM (2013) Application of Natural Blends of Phytochemicals Derived from the Root Exudates of Arabidopsis to the Soil Reveal That Phenolic-related Compounds Predominantly Modulate the Soil Microbiome. *Journal of Biological Chemistry* **288**: 4502-4512.
- Barea JM, Azcón R & Azcón-Aguilar C (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. *Antonie van Leeuwenhoek* **81**: 343-351.
- Barrios E (2007) Soil biota, ecosystem services and land productivity. *Ecological Economics* **64**: 269-285.
- Berruti A, Borriello R, Orgiazzi A, Barbera AC, Lumini E & Bianciotto V (2014) Arbuscular Mycorrhizal Fungi and their Value for Ecosystem Management. *Biodiversity: The Dynamic Balance of the Planet*, (Grillo O, ed.). Intech Open.
- Bougoure DS & Cairney JW (2005) Fungi associated with hair roots of *Rhododendron lochiaie* (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture independent molecular methods. *Environmental Microbiology* **7**: 1743-1754.
- Bünemann EK, Schwenke GD & Van Zwieten L (2006) Impact of agricultural inputs on soil organisms—a review. *Soil Research* **44**: 379-406.
- Dodd IC & Ruiz-Lozano JM (2012) Microbial enhancement of crop resource use efficiency. *Current Opinion in Biotechnology* **23**: 236-242.
- Eichhorn E, van der Ploeg JR & Leisinger T (1999) Characterization of a two-component alkanesulfonate monooxygenase from *Escherichia coli*. *Journal of Biological Chemistry* **274**: 26639-26646.
- Faye A, Dalpé Y, Ndung'u-Magiroi K, Jefwa J, Ndoye I, Diouf M & Lesueur D (2013) Evaluation of commercial arbuscular mycorrhizal inoculants. *Canadian Journal of Plant Science* **93**: 1201-1208.
- FDA (2011) BAM Appendix 2: Most Probable Number from Serial Dilutions. (Blodgett R, ed.).
- Fox A, Kwapinski W, Griffiths BS & Schmalenberger A (2014) The role of sulfur and phosphorus mobilizing bacteria in biochar induced growth promotion of *Lolium perenne*. *FEMS Microbiology Ecology* **90**: 78-91.
- Gahan J & Schmalenberger A (2015) Arbuscular mycorrhizal hyphae in grassland select for a diverse and abundant hyphospheric bacterial community involved in sulfonate desulfurization. *Applied Soil Ecology* **89**: 113-121.
- Gahan J, O'Sullivan O, Cotter P & Schmalenberger A (2021) The role of arbuscular mycorrhiza and organosulfur mobilizing bacteria in plant sulphur supply. *bioRxiv* 2021.2002.2008.429889.
- Gardes M & Bruns TD (1993) ITS primers with enhanced specificity for *Basidiomycetes*: application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118.

- Gentili F & Jumpponen A (2006) *Potential and possible uses of bacterial and fungal biofertilizers*. Haworth Press, New York.
- Gianinazzi S & Vosátka M (2004) Inoculum of arbuscular mycorrhizal fungi for
810 production systems: science meets business. *Canadian Journal of Botany* **82**: 1264-1271.
- Grayston SJ, Wang S, Campbell CD & Edwards AC (1998) Selective influence of plant
species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry* **30**: 369-
378.
- Helgason T, Daniell TJ, Husband R, Fitter AH & Young JPW (1998) Ploughing up the
815 wood-wide web? *Nature* **394**: 431-431.
- Helgason T, Merryweather JW, Denison J, Wilson P, Young JPW & Fitter AH (2002)
Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and
plants from a temperate deciduous woodland. *Journal of Ecology* **90**: 371-384.
- Hodge A & Fitter AH (2010) Substantial nitrogen acquisition by arbuscular mycorrhizal
820 fungi from organic material has implications for N cycling. *Proceedings of the National
Academy of Sciences* **107**: 13754-13759.
- Hodge A, Campbell CD & Fitter AH (2001) An arbuscular mycorrhizal fungus accelerates
decomposition and acquires nitrogen directly from organic material. *Nature* **413**: 297-299.
- Humphreys CP, Franks PJ, Rees M, Bidartondo ML, Leake JR & Beerling DJ (2010)
825 Mutualistic mycorrhiza-like symbiosis in the most ancient group of land plants. *Nature
Communications* **1**: 103.
- Jin H, Germida JJ & Walley FL (2013) Impact of arbuscular mycorrhizal fungal
inoculants on subsequent arbuscular mycorrhizal fungi colonization in pot-cultured field
pea (*Pisum sativum* L.). *Mycorrhiza* **23**: 45-59.
- Johansson JF, Paul LR & Finlay RD (2004) Microbial interactions in the
830 mycorrhizosphere and their significance for sustainable agriculture. *FEMS Microbiol
Ecology* **48**: 1-13.
- Jones DL, Hodge A & Kuzyakov Y (2004b) Plant and mycorrhizal regulation of
rhizodeposition. *New Phytologist* **163**: 459-480.
- Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa
835 K, Ishikawa A & Kawashima K (2000) Complete genome structure of the nitrogen-fixing
symbiotic bacterium *Mesorhizobium loti*. *DNA Research* **7**: 331-338.
- Kertesz MA & Mirleau P (2004) The role of microbes in plant sulphur supply. *Journal of
Experimental Botany* **55**: 1939-1945.
- Kertesz MA, Fellows E & Schmalenberger A (2007) Rhizobacteria and plant sulfur
840 supply. *Advances in Applied Microbiology* **62**: 235-268.
- King JE & Quinn JP (1997) The utilization of organosulphonates by soil and freshwater
bacteria. *Letters in Applied Microbiology* **24**: 474-478.
- Klironomos JN & Hart MM (2002) Colonization of roots by arbuscular mycorrhizal fungi
845 using different sources of inoculum. *Mycorrhiza* **12**: 181-184.
- Lane DJ (1991) *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons,
Chichester, UK.
- Ludwig W, Strunk O, Westram R, *et al.* (2004) ARB: a software environment for
sequence data. *Nucleic Acids Research* **32**: 1363-1371.
- Lynch JM & Whipps JM (1990) Substrate flow in the rhizosphere. *Plant Soil* **129**: 1-10.
- Marschner H & Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. *Plant and Soil*
850 **159**: 89-102.
- McGonigle TP, Miller MH, Evans DG, Fairchild GL & Swan JA (1990) A new method
which gives an objective measure of colonization of roots by vesicular-arbuscular
855 mycorrhizal fungi. *New Phytologist* **115**: 495-501.

- McGrath SP, Zhao FJ & Blake-Kalff MM (2003) History and outlook for sulphur fertilizers in Europe. Vol. Proceedings 497. York, UK.
- Muyzer G, De Waal E & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.
- Öpik M, Moora M, Liira J & Zobel M (2006) Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. *Journal of Ecology* **94**: 778-790.
- Peix A, Rivas-Boyer AA, Mateos PF, Rodriguez-Barrueco C, Martinez-Molina E & Velazquez E (2001) Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber conditions. *Soil Biology and Biochemistry* **33**: 103-110.
- Pellegrino E, Turrini A, Gamper HA, Cafa G, Bonari E, Young JPW & Giovannetti M (2012) Establishment, persistence and effectiveness of arbuscular mycorrhizal fungal inoculants in the field revealed using molecular genetic tracing and measurement of yield components. *New Phytologist* **194**: 810-822.
- Plenchette C, Clermont-Dauphin C, Meynard JM & Fortin JA (2005) Managing arbuscular mycorrhizal fungi in cropping systems. *Canadian Journal of Plant Science* **85**: 31-40.
- Ray DK, Mueller ND, West PC & Foley JA (2013) Yield trends are insufficient to double global crop production by 2050. *PloS One* **8**: e66428.
- Read DJ & Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems—a journey towards relevance? *New Phytologist* **157**: 475-492.
- Reasoner DJ & Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* **49**: 1-7.
- Rowe HI, Brown CS & Claassen VP (2007) Comparisons of mycorrhizal responsiveness with field soil and commercial inoculum for six native montane species and *Bromus tectorum*. *Restoration Ecology* **15**: 44-52.
- Schmalenberger A & Kertesz MA (2007) Desulfurization of aromatic sulfonates by rhizosphere bacteria: high diversity of the *asfA* gene. *Environmental Microbiology* **9**: 535-545.
- Schmalenberger A & Noll M (2010) Shifts in desulfonating bacterial communities along a soil chronosequence in the forefield of a receding glacier. *FEMS Microbiol Ecol* **71**: 208-217.
- Schmalenberger A, Schwieger F & Tebbe CC (2001) Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. *Applied and Environmental Microbiology* **67**: 3557-3563.
- Schmalenberger A, Drake HL & Kuesel K (2007) High unique diversity of sulfate-reducing prokaryotes characterized in a depth gradient in an acidic fen. *Environmental Microbiology* **9**: 1317-1328.
- Schmalenberger A, Telford A & Kertesz MA (2010) Sulfate treatment affects desulfonating bacterial community structures in *Agrostis* rhizospheres as revealed by functional gene analysis based on *asfA*. *European Journal of Soil Biology* **46**: 248-254.
- Schmalenberger A, Hodge S, Hawkesford MJ & Kertesz MA (2009) Sulfonate desulfurization in *Rhodococcus* from wheat rhizosphere communities. *FEMS Microbiol Ecol* **67**: 140-150.
- Schmalenberger A, Hodge S, Bryant A, Hawkesford MJ, Singh BK & Kertesz MA (2008) The role of *Variovorax* and other *Comamonadaceae* in sulfur transformations by microbial

- 905 wheat rhizosphere communities exposed to different sulfur fertilization regimes.
Environmental Microbiology **10**: 1486-1500.
- Schmalenberger A, Hodge S, Bryant A, Hawkesford MJ, Singh BK & Kertesz MA (2008) The role of *Variovorax* and other Comamonadaceae in sulfur transformations by microbial wheat rhizosphere communities exposed to different sulfur fertilization regimes.
910 *Environmental Microbiology* **10**: 1486-1500.
 - Simon L, Lalonde M & Bruns TD (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. *Applied and Environmental Microbiology* **58**: 291-295.
 - Smit AL, Bindraban PS, Schröder JJ, Conijn JG & Van der Meer HG (2009) Phosphorus
915 in agriculture: global resources, trends and developments. *Report to the Steering Committee Technology Assessment of the Ministry of Agriculture, The Neetherlands, Wageningen*.
 - Treseder KK (2013) The extent of mycorrhizal colonization of roots and its influence on plant growth and phosphorus content. *Plant and Soil* **371**: 1-13.
 - 920 • Tunney H, Kirwan L, Fu W, Culleton N & Black AD (2010) Long-term phosphorus grassland experiment for beef production—impacts on soil phosphorus levels and liveweight gains. *Soil Use and Management* **26**: 237-244.
 - Van Der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A & Sanders IR (1988) Mycorrhizal fungal diversity determines plant
925 biodiversity, ecosystem variability and productivity. *Geological Society of America Bulletin* **100**: 912-927.
 - Vermeij P, Wietek C, Kahnert A, Wüest T & Kertesz MA (1999) Genetic organization of sulphur-controlled arylsulphonation in *Pseudomonas putida* S-313. *Molecular Microbiology* **32**: 913-926.
 - 930 • Vilarino A, Frey B & Shüepp H (1997) MES [2-(N-morpholine)-ethane sulphonic acid] buffer promotes the growth of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices* in an alkaline sand. *Biology and Fertility of Soils* **25**: 79-81.
 - Wang FY, Tong RJ, Shi ZY, Xu XF & He XH (2011) Inoculations with arbuscular mycorrhizal fungi increase vegetable yields and decrease phoxim concentrations in carrot
935 and green onion and their soils. *PloS One* **6**: e16949.
 - White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* 315-322.
 - Zhao FJ, Lehmann J, Solomon D, Fox MA & McGrath SP (2006) Sulphur speciation and
940 turnover in soils: evidence from sulphur K-edge XANES spectroscopy and isotope dilution studies. *Soil Biology and Biochemistry* **38**: 1000-1007.

6. Acknowledgements

The authors would like to thank the staff of Teagasc, Johnstown Castle, for providing the soil
945 used in this experiment and FP7 People (CIG no. 293429) for funding this project.