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

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Abstract

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

Introduction

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

has been reported that biostimulants are most efficacious when used in conjunction with integrated nutrient management regimes (Rouphael *et al*., 2015).

One of the most unclear aspects of biostimulant activity is how to identify modes of action by which plant growth may be enhanced. In addition to increasing levels of plant-available nutrients in the rhizosphere, both organic and inorganic fertiliser amendments affect the community structure and composition of soil microorganisms (Marschner *et al*., 2003). As the structure and composition of rhizosphere microbes change, plant growth may be correspondingly influenced. A variety of factors have been known to be responsible for this, including increased nutrient turnover, induced disease resistance, competing for active sites on root tissue, interfering with pathogen signalling hormones, and disease suppression (Storer *et al*., 2016), which are all very much subject to the seasonal effects and the timing of fertiliser application (Di Salvo *et al*., 2018). Evidence for a variety of fungi-derived plant growth-promoting effects is being increasingly documented, and which may include imprpved root morphology and increased plant nutrition through phosphorus mobilisation (Sanchez-Evesta *et al*., 2016). Rouphael *et al*. (2015) identify the need for research which improves understanding of interaction between fungal species, crops and environment for the purposes of improving the efficacy of proposed biostimulant formulations. The method used in the present study seeks to quantify morphological differences between plants treated with biostimulants and then contrast this with plant-available P in treated soils, and in so doing isolating the stages of the mode of action responsible for plant growth-promotion. The approaches used to characterise the phenotypes of a given species (phenomics) have been developed to overcome restrictions of traditional plant morphology measurement methods (Duan *et al*., 2018), and techniques now include novel image sensors, imaging analysis and robotics. The capability of this infrastructure to facilitate high-throughput and large-scale, non-invasive phenotyping may be used in addition to reduce the time and inaccuracy of traditional measurement methods, which are often manual. Visible light imaging technology, due to low cost and ease of implementation, is used widely for plant imaging. Through recording similar wavelength perception as in human vision (400-700 nm), 2D photography can detect useful information for the analysis of leaf biomass, yield and leaf morphology. In addition, Rouphael *et al*. (2015) highlighted the importance of identifying molecular mechanisms responsible for plant growth-promoting effects in commercial biostimulants. Owen *et al*. (2015) observe the complexities involved in determining whether the presence of a biostimulant is efficacious in crop growth-promotion, with interactions between microbial communities and crops varying depending on species composition, soil nutrient regime, temperature, pH, seasonality, and organic inputs.

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

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

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

the persistence, degradability, proliferation and spread of biostimulants are also largely unknown (Hart *et al*., 2018).

This study uses a number of approaches to address this knowledge gap. The literature has focused to a large extent on AMF species for plant growth promotion (Fester and Sawers, 2011; Smith and Read, 2010; Zuccaro *et al*., 2014), and other fungal phyla have been relatively unexplored in terms of commercial exploitation, and increasing evidence is emerging that the supposed benefits of attempting to manipulate AMF abundance in agricultural environments have been greatly overstated (Ryan and Graham, 2018). It has been suggested above that biostimulants may affect crops by different mechanisms and species to the intended ones, and in the case of the AMF biostimulant used in this study, initial investigation using simple culturing and molecular diagnostic methods (unpub.) revealed the presence of a number of fungal species from other phyla which were likely included in the biostimulant as a result of the culturing process. It is possible that purported growth improvements observed in crops treated with this biostimulant may not be due to mycorrhizal symbiosis, but could be due to other fungal taxa with lesser known effects (Detheridge *et al*., 2016). A means of investigating this would be to inoculate a non-mycorrhizal species with this biostimulant and examine the behaviour of other microbes in relation to plant P uptake, and this approach has been undertaken in this study, with a non-mycorrhizal experimental crop (*Arabidopsis thaliana)* used to examine the extent to which biostimulant efficacy is due to specified properties. The experimental method sought to do this by measuring morphological variables associated with crop performance, and measured P uptake to ascertain to what extent mineral nutrition was influenced by the experimental conditions. The choice of *Arabidopsis* as a model plant in the present study has been selected on account of its physiological relationship to oilseed rape (Santaniello *et al*., 2012). Rosettes are measured as opposed to the entire plant structure, which is intended to reduce the issues associated with sideways-positioned cameras in 2D phenomics imaging. The study involves autoclaved (sterilised) controls of all three biostimulants, allowing the effects of the microorganisms used in these formulations to be examined for their efficacy. The aim of the present study is to develop the method further by including sterilised controls of all biostimulants used.

Many factors influence the structure of fungal communities, including nutrient availability, pathogen presence in the rhizosphere, availability of active sites on root tissue, but mainly pH (Tian *et al*., 2017). The evidence of fungi-derived plant growth-promoting effects continues to be documented, and include increased root proliferation and surface area and improved plant nutrition through phosphorus mobilisation (Sanchez-Evesta *et al*., 2016). As well as bacteria, fungi can solubilise and chelate inorganic nutrients, allowing them to be available for plant uptake (Zhang *et al*., 2014). Detheridge *et al*. (2016) discuss the need to understand the

complexities of the interactions between plants, the soil and microbes, their roles in plant nutrition, soil function and how they may be affected by soil nutrient status, temperature, pH and organic inputs as a result of agricultural management practices. Organic and inorganic fertiliser amendments are well-understood to affect the community structure of soil microorganisms (Marschner *et al*., 2003), which has resulting implications on crop growth. Rouphael *et al*. (2015) identify the need to research the interaction between fungi, crops and abiotic variables. Similarly, Dias *et al.* (2015) highlight that a better understanding of the role of microorganisms in plant nutrition and nutrient cycles in agricultural contexts is needed, and stress that the interactions between fungi, plants and other soil microbial communities are of particular importance. Generating useful results at the appropriate large data scales is becoming increasingly possible through high-throughput metagenomics (Carbonetto *et al*., 2014), and in so doing, allowing us to better isolate the modes of action responsible for plant growth-promotion. Rouphael *et al*. (2015) also identify the importance of elucidating the molecular mechanisms involved in plant growth-promoting effects in commercial biostimulants. The approach used in this study involved examining fungal DNA in the rhizosphere of treated plants, which allows us to suggest which fungal species persist in the soil after biostimulants have been applied, and the nature of fungal community structure after inoculation. This information is essential for the purposes of determining the persistence and longevity of the molecular mechanisms involved in plant growth promotion following biostiumlant application (Rouphael *et al*., 2015).

Materials and methods

Experiment design

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

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

Table 1a: Descriptions of treatments, composition and function of commercial biostimulants used in study. All biostimulants were stored and applied in powder form.

Biostimulant	Acronym	Carrier	Biology
Biagro Brassika + P	KGB+P	Soluble powder	*B. amyloliquefaciens, B. subtilus, B. lichenoformis, B. circulans, B. megatererium, B. pumilus, B. coagulans*
Biagro Brassika - P	KB-P		
Sterilised Biagro Brassika + P	KB(S)+P		*Sterilised*
Sterilised Biagro Brassika - P	KB(S)-P		
Humostart rhizobium + P	UR+P	Micro-ground DAP plus zinc, suspended in mono-potassium phosphate and di-	Several phosphate-mobilising & N fixing genera of bacteria (listed as *Bacillus, Paenibacillus, Azotobacter, Azospirillum* and *Beijerinckia*)
Humostart rhizobium – P	UR-P		

Sterilised Humostart rhizobium + P	UR(S)+P	potassium phosphate with zeolite	*Sterilised*
Sterilised Humostart rhizobium – P	UR(S)-P		
Humostart mycorrhizae + P	UM+P	Micro-ground DAP plus zinc with zeolite and AMF blended through	Unconfirmed but very probably contains *Trichoderma, Glomus interadices, G. mosseae, G. aggregatum* and *G. etunicatum*
Humostart mycorrhizae - P	UM-P		
Sterilised Humostart mycorrhizae + P	UM(S)+P		*Sterilised*
Sterilised Humostart mycorrhizae - P	UM(S)-P		

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

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198 **Table 1b**: Elemental composition (%) of selected commercial biostimulants (Medac Ltd,
199 2019). Various reasons prevent disclosure of the full composition all biostimulants used in this
200 study

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

UM	0.17	4.31	10.59	<0.10	0.25	75.49	4.69

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

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

To avoid confusion, all mentions of "soil" in this report refer to the substrate described here.

Data collection and analysis

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

This process enabled the area of experimental crop rosettes to be calculated, as well as the perimeter. Area was selected as the most informative overall measure of growth that could be inferred from morphological measurements, as it could be directly linked to biomass (whereas perimeter, circularity, symmetry and eccentricity parameters cannot). The smallest convex hull which contained each rosette was detected using OpenCV, which calculated pixel counts for each plant. From these values, the ratio of rosette area to convex hull area was used to calculate compactness scores. The parameters of rosette perimeter, area and extracted

convex hulls were used for calculations of further rosette morphometric parameters (roundness1, roundness2, isotropy, eccentricity, rotational mass symmetry (RMS), and slenderness of leaves (SOL)) (PlantScreen analyzer™, PSI, Czech R.).

To characterise detailed morphology of treated plants, morphological parameters were grouped into four general categories based on the general characteristics they measure, following the approach described in Pavicic *et al*. (2017). These categories were growth measurements, circularity, symmetry and centre distance. These parameters were then compared over time. Growth measurements consisted of area and perimeter, Roundness1 and roundness2 and isotropy constituted the circular parameters. Roundness1 refers to the comparison between measured rosette area and a perfect circle with the same perimeter. These variables are controlled by slenderness and perimeter of the leaves and petiole length. In wild plants, roundness1 usually measures 0.1 and 0.5 (Pavicic *et al*., 2017), and a perfect circle has a value of 1. Roundness2 compares the area of rosette convex hulls with plant perimeter, and wild plants are reported to measure between 0.7 and 1.0 for this ratio. Isotropy measurements involve imposing a polygon over the rosette, and therefore behaves in a similar manner to roundness 2 but with a decrease in tendency. Circularity parameters, while not directly linked to biomass, can nevertheless provide information on the onset of the transition from vegetative to reproductive growth, the determining of which can have major effects on biomass accumulation (Camargo *et al*., 2016).

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

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

Phosphorous measurements

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

Substrate preparation and DNA extraction

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

Amplification was performed in a 50 µl Polymerase Chain Reaction (PCR) using Promega GoTaq G2, DNA polymerase (Promega, Madison USA). Each reaction contained 250 nM of each primer, 1 mg ml-1 BSA, 200 µ M dNTPs and 0.5 U of DNA polymerase in the buffer. The

primer D1F2 was linked at the 5' end to the IonTorrent adapter sequence (CCATCTCATCCCTGCGTGTCTCCGAC), a TCAG key and an IonXpress Barcode. Primer NLC2AF was linked at the 5' end to an Ion Torrent adapter sequence (CCTCTCTATGGGCAGTCGGTGAT). The PCR conditions were 94 °C for 5 min (initial denaturation) followed by 30 cycles at 94 °C, 30 s (denaturation); 52 °C, 30 s (annealing); 72 °C, 30 s (extension) and a final extension step at 72 °C for 5 min. The PCR reactions were cleaned using spin columns (NBS Biological, Huntingdon UK) and the amplified DNA was quantified using NanoDrop (NanoDrop Products, Wilmington USA). Emulsion PCR was carried out using Ion-Torrent 200 bp template kit using the Ion Torrent One Touch 2 system following the manufacturer's instructions. Amplified sequence particles were enriched using One Touch ES to remove non-template particles, and were then sequenced on "316" (100 Mbp) microchips using the Ion Torrent Personal Genome Machine (Life Technologies, Waltham USA).

4Sequence data processing

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

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

Equation 1: Shannon diversity index

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

Equation 2: Simpson diversity index

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

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

Statistical analysis

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

data checked for normality (Shapiro-Wilk). All results were considered significant at the $p < 0.05$ level.

Results

Multivariate Analysis of Variance (MANOVA) analysis demonstrated that both P amendments and sterilising biostimulants had highly significant effects (MANOVA, $p < 0.0001$). Two-sample Kolmogorov-Smirnov tests between the Control+P treatment and the other biostimulant treatments revealed significant differences between treatments across all variables (Table 2).

Table 2: Summary of P values between Two-sample Kolmogorov-Smirnov tests carried out between all biostimulant treatments and the Control+P treatment across the nine variables measured. Asterisks denote levels of significance. P-values below 0.05 are flagged with one asterisk (*), p-values below 0.01 are flagged with two asterisks (**), p-values below 0.001 are flagged with three asterisks (***)

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

Growth measurements

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

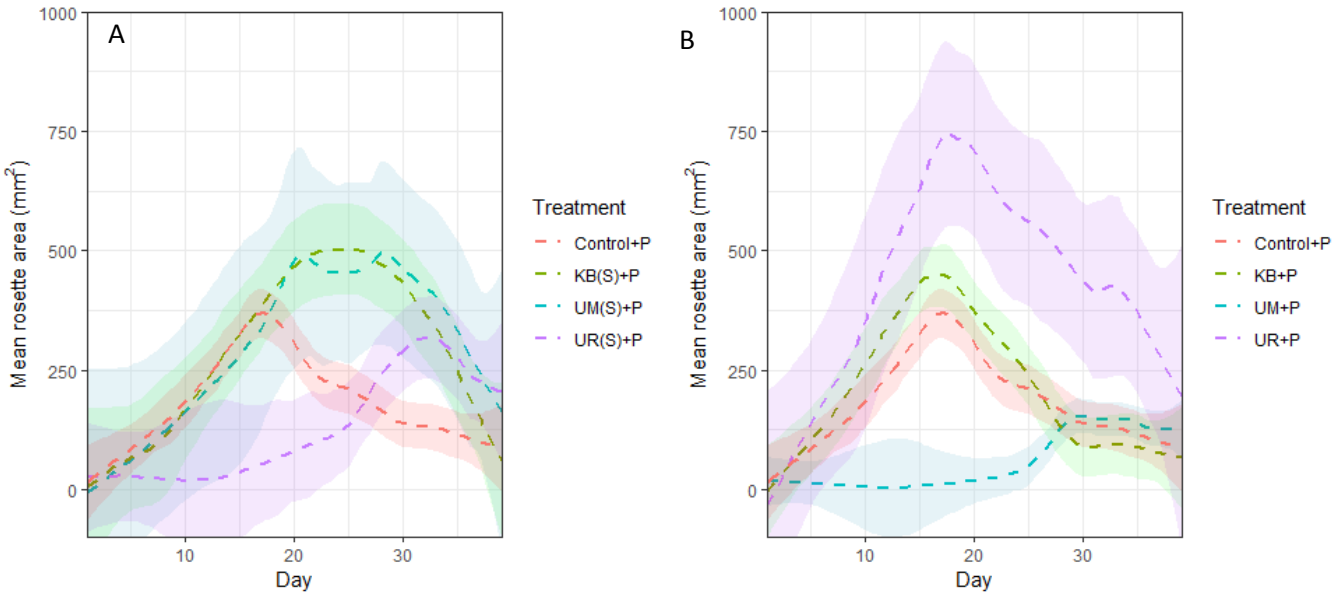


Figure 1: Growth measurements for rosette area from day 1 to 40 of P-amended treatments for **(A)** Sterilised biostimulants and **(B)** live biostimulants. Curves denote daily mean; shaded areas denote +/- SE, 95 % confidence interval. n = 10 plants

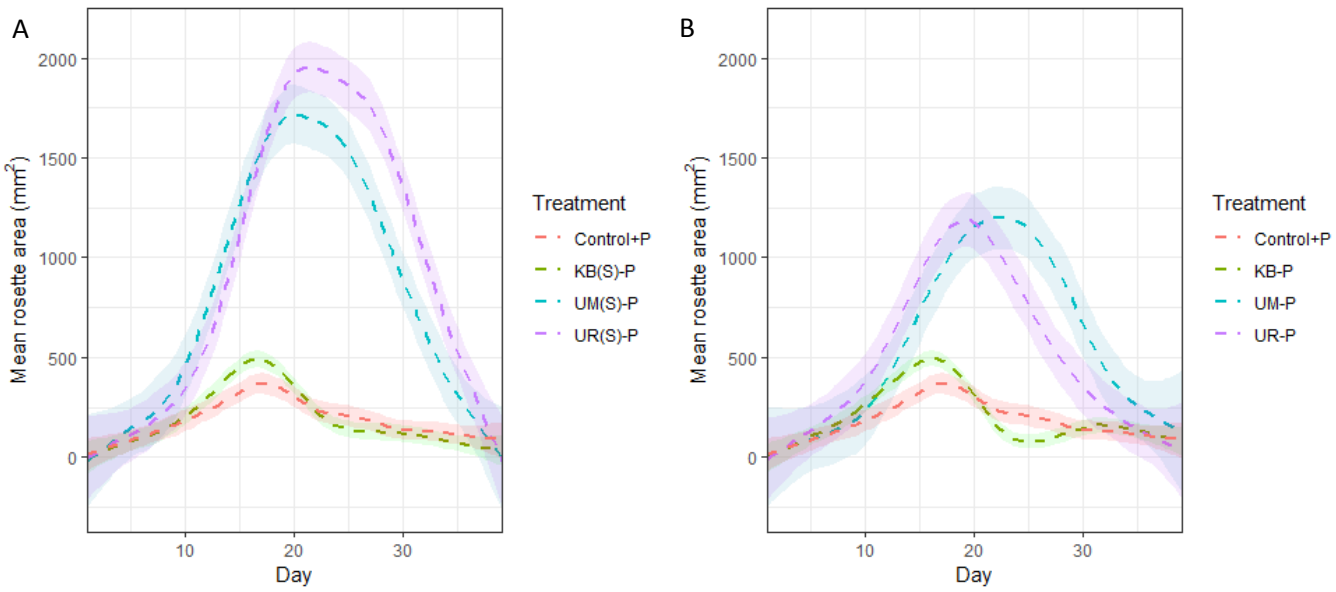


Figure 2: Growth measurements for rosette area from day 1 to 40 for non-P amended treatments for **(A)** Sterilised biostimulants and **(B)** live biostimulants. Curves denote daily mean; shaded areas denote +/- SE, 95 % confidence interval. n = 10 plants

Morphological measurements

Morphological data for the circularity parameters of roundness, roundness 2 and isotropy were also analysed (Figure 3). Live UM treated plants in P-amended soils showed the highest overall roundness over the total experimental period and in comparison to the controls and the other two biostimulants, and also had the greatest uncertainty. Live UR treated plants in P-amended soils showed the least decrease in roundness over the experimental period, while both live and sterilised KB treatments in non-P treated soils showed the steepest decrease in roundness. Live UR treated plants in P-amended soils also had significantly lower isotropy than the controls and other treatments for the first three weeks of the experimental period. In non-P amended soils, all three biostimulant treatments measured faster declines in isotropy than the controls in both P-amended and non-P amended soils. The morphological parameters that describe symmetry are eccentricity and rotational mass symmetry. For eccentricity, KB treated plants showed the greatest increases in P-treated and non-P treated plants whether sterilised or not. Live UM treated plants showed considerable variability in eccentricity for the first three weeks of the experimental period in P-amended soils. Sterilised UR and UM treatments resulted in less eccentricity than the controls in non-P amended soils, but live treatments produced similar curves to the control. For rotational mass symmetry, sterilised UR treatments in P-amended soils did not follow the usual pattern of a dip followed by a peak, but

instead remained relatively constant throughout the growing period (with higher uncertainty than other treatments). Non-sterilised UM followed a similarly aberrant pattern in P-amended soils, remaining relatively constant until the end of the third week of the experimental period. In non-P amended soils, the dip in the curves for sterilised UR and UM was significantly greater than the controls and KB, but live biostimulants produced similar curves to the control. The final morphological parameters analysed were compactness and slenderness of leaves (SOL), which relate to centre distance. In P-amended soils, sterilised and live biostimulants showed a decay in compactness closely resembling the controls, but in non-P amended soils, both live and sterilised UR and UM treatments took longer for compactness to decay than in the controls or KB. Similarly to results seen in the other variables, sterilised UM in P-amended soils had a significantly greater peak in the curve for slenderness of leaves than the control. The other two treatments also exhibited a peak in SOL during the fourth week of the experimental period. Live UR exhibited significantly higher SOL than the other treatments in P-amended soils. In non-P amended soils, UR and UM treatments (both live and sterilised) resulted in significantly higher peaks for SOL than KB and the controls, although sterilised treatments produced significantly higher peaks than live biostimulants.

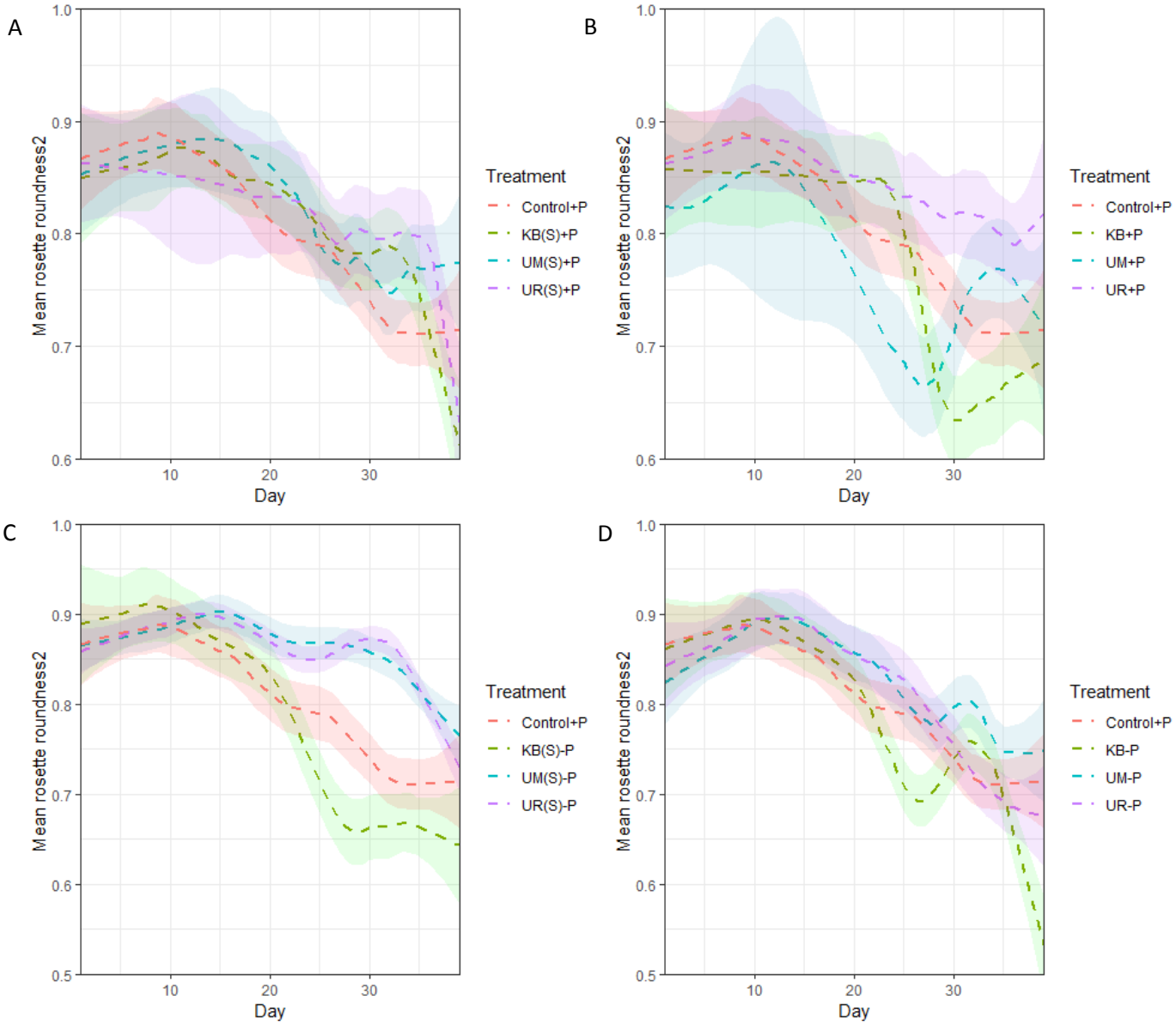


Figure 3: Circularity measurements for rosette roundness2 from day 1 to 40. **(A)** Sterilised biostimulants with P amendments, **(B)** live biostimulants with P amendments, **(C)** Sterilised biostimulants without P amendments, and **(D)** live biostimulants without P amendments. Curves denote daily mean; shaded areas denote +/- SE, 95 % confidence interval. n = 10 plants

Phosphorus measurements

There was significantly less plant-available P observed in soil treated with three biostimulants (Figure 4, KB-P, KB(S)-P, Control-P). Boxes show extent of upper/lower quartiles around the mean, and whiskers show upper and lower extent of data. Asterisks denote levels of significant variation to the Control+P reference group. P-values below 0.05 are flagged with one asterisk (*), p-values below 0.01 are flagged with two asterisks (**), p-values below 0.001 are flagged with three asterisks (***), and p-values below 0.0001 are flagged with four asterisks (****). Grey circles denote extreme outliers.

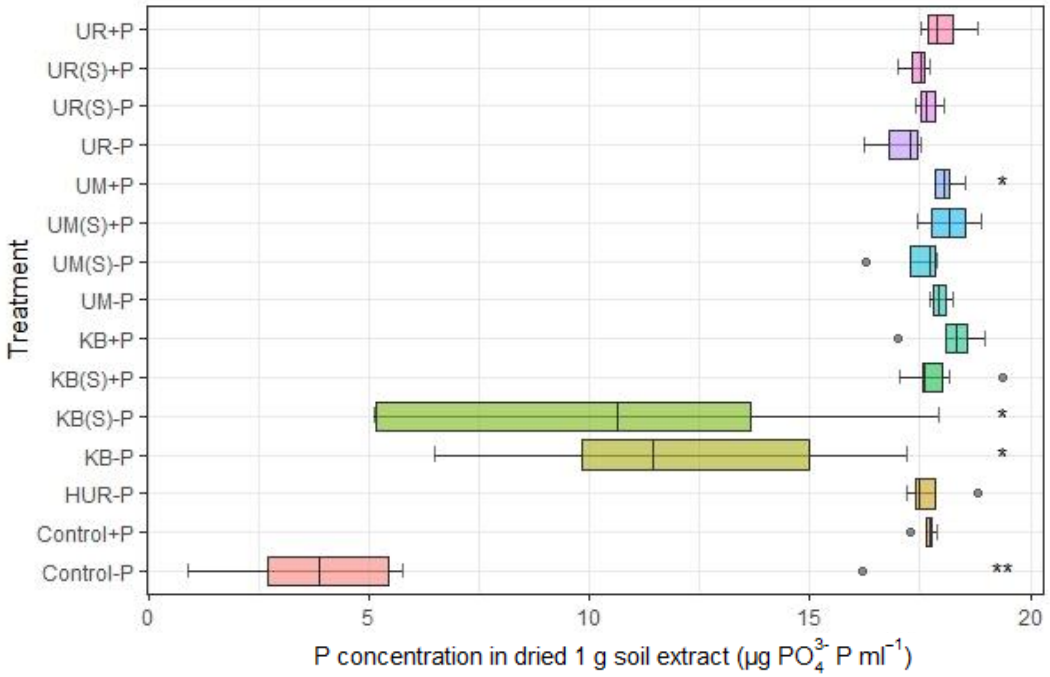


Figure 4: Summary of mean PO_4^{3-} (plant-available phosphorus) concentrations obtained from GEN5™ microplate analysis of ten replicates of each biostimulant treatment in 1 g dried soil extracts

After removing results from these three treatments showing significantly lower plant-available P than the other treatments, differences between the remaining treatments became easier to visualise (Figure 5). Soil P concentrations of these remaining treatments were fairly similar, with the exception of the UM+P treatment, which was significantly higher than the Control+P reference group. To some extent, these results are fairly close to what was expected, in that the Control-P treatments did not receive RB209 levels of P and was therefore severely P limited, and neither did the live and sterilised KB-P treatments which had less than 3% PO_4^{3-} levels in the carrier substrate. By comparison, the P levels of the live and sterilised UM and UR treatments were expected to be higher as their carrier substrates contained over 70% PO_4^{3-} by elemental composition. However, there was very little difference between the plant available P levels of the soils treated with KGB (live and sterilised) biostimulants and the UR and UM treatments whether the latter were P amended or not. Therefore, since the latter treatments introduced substantially more P into the soils than KGB treatments through the carrier substrate (~50%), the surplus P must either have been leached from the soil or taken up by the plants.

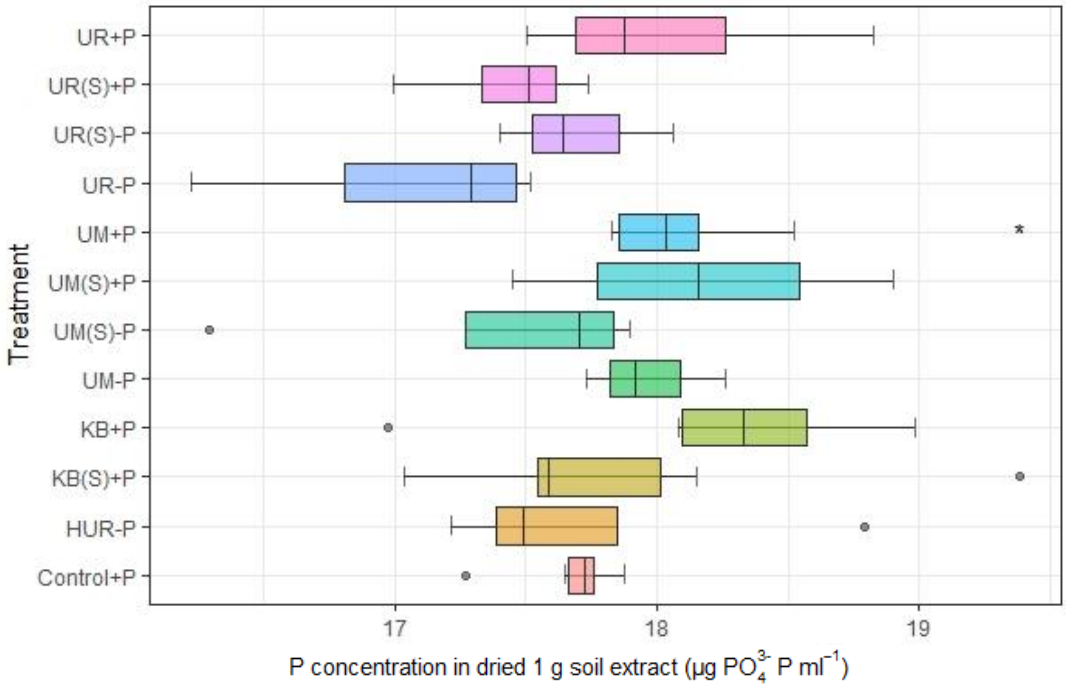
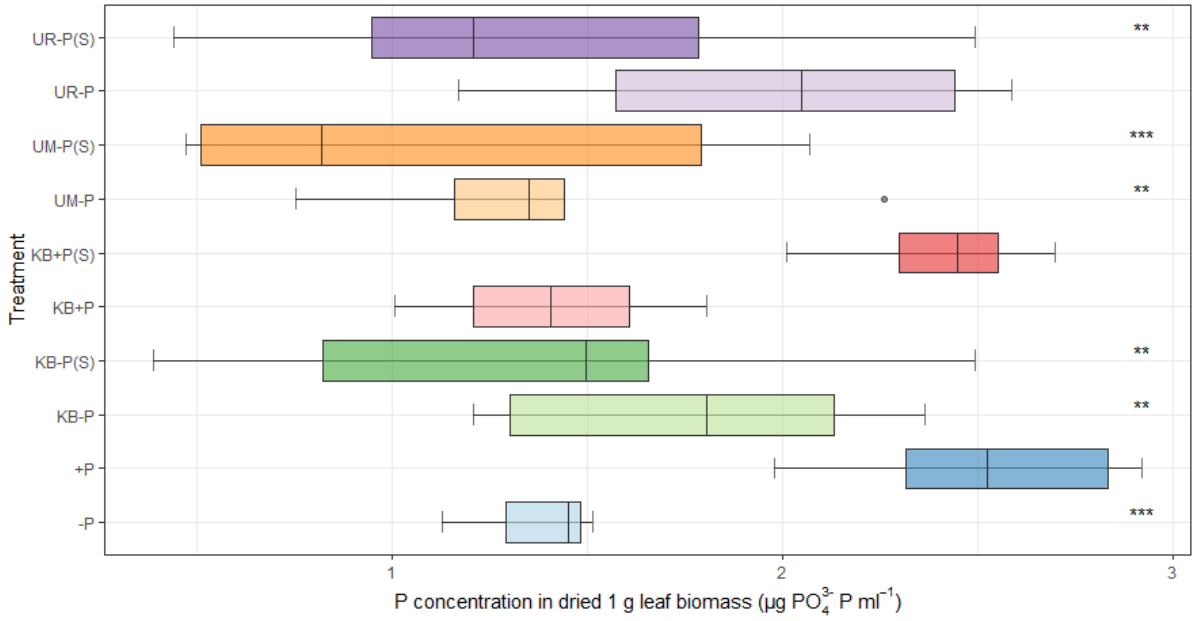


Figure 5: Summary of mean PO_4^{3-} (plant-available phosphorus) concentrations obtained from GEN5™ microplate analysis of the replicates of each biostimulant treatment in 1 g dried soil extracts minus the three treatments showing significantly less than the others. Some significant differences between these remaining results are now easier to visualise as opposed to before removing these three treatments

Lower levels of P were measured in the leaf tissue of plants receiving biostimulant treatments than in the P-amended control, which actually had the highest mean leaf tissue P observed across all treatments (Figure 6). This could be evidence of some metabolic cost to the plants from the biostimulant treatments, although it is unclear whether this would be a result of a direct or an indirect processes (such as more carbon exuded to the soil that impacted on the synthesis of ATP, for example). In P-limited soils, plants treated with live biostimulants had higher mean leaf tissue P than sterilised biostimulants, although this was not significant. In P-amended soils, it was the sterilised biostimulants that were more associated with elevated plant tissue P than the live treatments, and was significantly higher in the KGB treatment. In P-limited soils, both the live and the sterilised KGB treatments were associated with significantly lower leaf tissue P than the P amended control, which was not suprising considering how much less plant available P was present in the soils of these treatments (Figure 4). The levels of leaf tissue P in plants treated with the live UR-P biostimulant were not significantly lower than the control, however neither were the leaf tissue P levels of the plants treated with the sterilised KB+P treatment. Overall, no evidence from these findings would be indicative of microbially enhanced crop P uptake.



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490 **Figure 6**: Summary of mean PO_4^{3-} concentrations obtained from GEN5™ microplate analysis
491 of the replicates of each biostimulant treatment in 1 g dried leaf extracts. Data could not be
492 obtained from all treatments because plants that underwent some treatments did not have
493 harvestable biomass by the end of the experimental cycle.

494

Fungal community structure

Across the 14 substrate samples which were analysed, the mean number of sequences obtained after quality control was 28,172 per sample, the lowest being 15,598 (Table 3). Of these, >86 % were fungal, the remainder being mostly belonging the genera *Cercomonas* and *Chlorophyta* incertae sedis (of domains Rhizaria and Viridiplantae), which accounted for 23 % and 19 % respectively of the non-fungal sequences cross all samples. General statistics from sequencing run are presented in Table 4.

Table 3: Summary statistics for sequencing run. All diversity indexes are derived from the equations for Shannon and Simpson indexes, which are given in Equations 1 and 2.

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

Ascomycota were the most abundant fungi (mean 56 %; range 17–84 %), followed by Basidiomycota (mean 7 %; range 1–22 %). More than 85 % of fungal sequences were identified to family and more than 65 % identified to genus. The most abundant taxa were the saprophytes *Mortierella parvispora* (mean 22 %; range 3–77 %), *Pseudogymnoascus* (mean 11 %; range 1–26 %), and the ectomycorrhizal *Sphaerosporella* (mean 11 %; range 0–51 %). The most abundant taxa across all samples are displayed in and Figure 7, with Figure 8 displaying Ascomycota and Basidiomycota abundance.

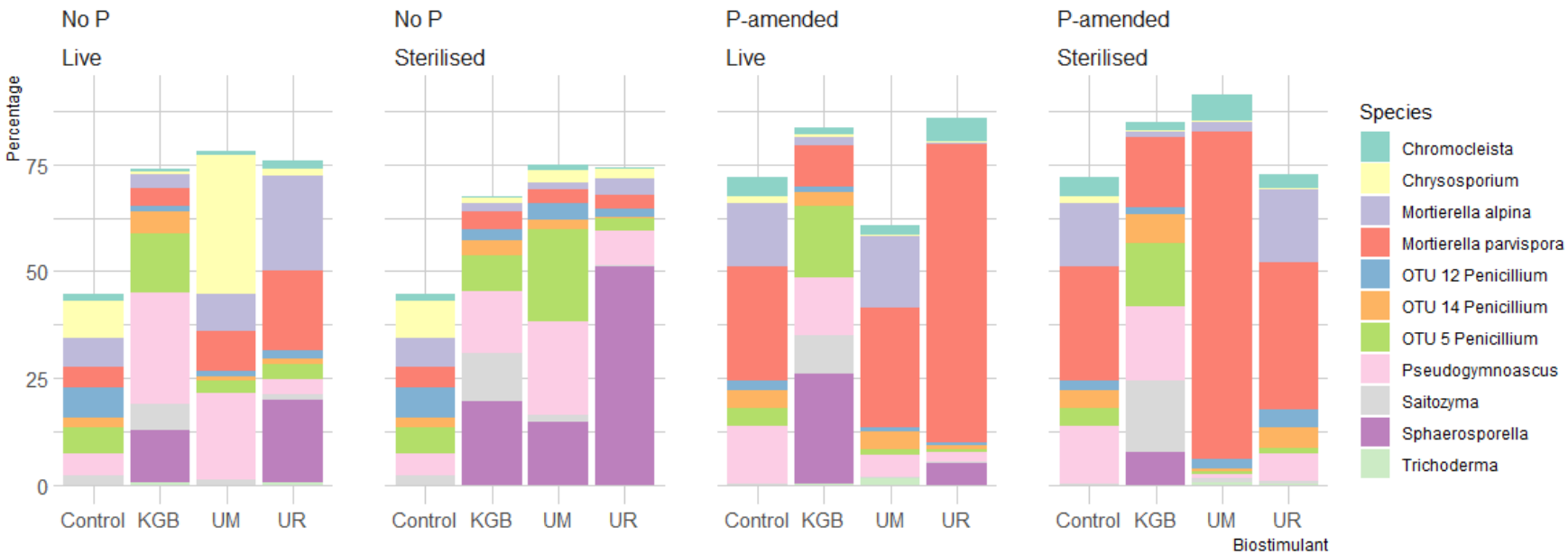

Figure 7: Stacked barchart showing mean abundance of the top ten most commonly occurring taxa across the three live and sterilised biostimulant treatments and controls in P-amended and non-P amended substrate (KGB: BiagroBrassiKa, UM: Umostart Mycorrhizae, UR: Umostart Rhizobium)

Table 4: Top 20 fungal sequences across all treatments and the mean, maximum (max) and minimum (min) relative abundance (%) of the Ion Torrent™ output, determined by overall cumulative abundance

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

Biostimulant effects

Biostimulant treatment was found to have had a significant effect on Basidiomycota abundance (ANOVA, p = 0.01) (Figure 3). KGB treatments had significantly higher Basidiomycota abundance than UM or UR treatments (ANOVA, p = 0.02 for both pairs). In particular, *Saitozyma* abundance was significantly affected (ANOVA, p = 0.0001). The treatment KGB was associated with increases in *Saitozyma* abundance that were not affected by sterilising the biostimulant or by P-amendments (post-hoc Tukey; KGB-Control, p = 0.0004; UM-KGB, p = 0.0003 and UR-KGB, p = 0.0003). A combined effect of phosphorus and biostimulant treatment was found to have a significant effect on *Trichoderma* (Two-way ANOVA, p = 0.04), which was more abundant in P-amended substrate treated with UM than in the non-P amended treatments. Sterilised treatments which had no P-amendments produced only trace abundance of Trichoderma OTUs. NMDS ordination (Figure 8) was able to discern separation in fungal community structure between the biostimulant treatments

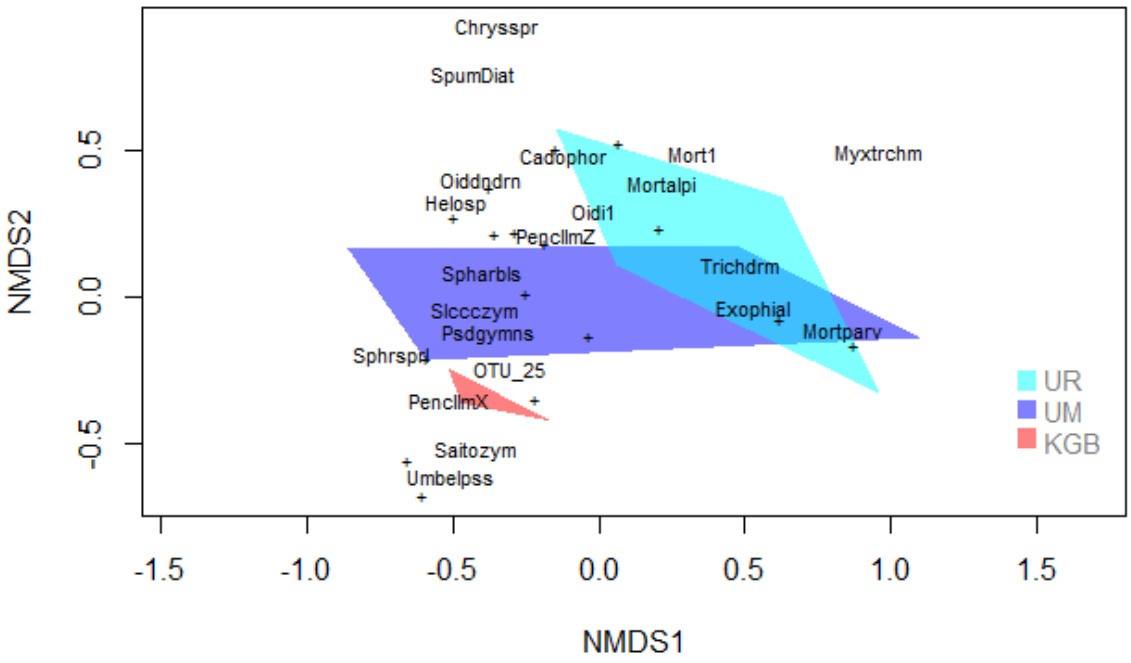

Figure 8: Non-metric multidimensional scaling analysis of fungal community data across all three biostimulant treatments. Some separation is apparent between convex hulls drawn between the UM and UR treatment community data, and clear separation is apparent between both and the KGB treatment community data

Figure 9: Stacked barchart showing mean abundance of the top eight most commonly occurring Ascomycota (a) and top five most commonly occurring Basidiomycota (b) across the three live and sterilised biostimulant treatments and controls in P-amended and non-P amended substrate. Some taxa associated with plant growth promotion are clearly represented and show considerable variation between treatments (particularly *Trichoderma, Saitozyma* and *Solicoccozyma*)

Phosphorus effects

Phosphorus amendments were found to have an overall significant effect on fungal community taxa abundance (MANOVA, p = 0.008), and principle coordinates analyses of fungal community structure was able to discern clear separation (Figure 10). In order to identify which fungal species and which fungal functional groupings were most affected by different P-amendments and whether this varied depending on live or sterilised biostimulant treatments, the relative proportions of the top 30 taxa were analysed for variance. Phosphorus amendments were significantly associated with an increase in the abundance of the overall most abundant taxon *Mortierella parvispora* (ANOVA, p = 0.005). P-amendments were also significantly associated with increases in the abundance of *Chromocleista* (ANOVA, p = 0.0005) that were not associated with biostimulant treatment or whether the treatment was live or sterilised (Tukey, post hoc). P-amendments had a significant effect on Mucormycota taxa abundance (ANOVA, p = 0.0005), which largely consisted of species from the *Mortierella* genus. Ascomycota abundance was significantly reduced by P-amendments (ANOVA, p = 0.04), although the Ascomycota community structure of KGB treated substrate remained relatively consistent under all treatments.

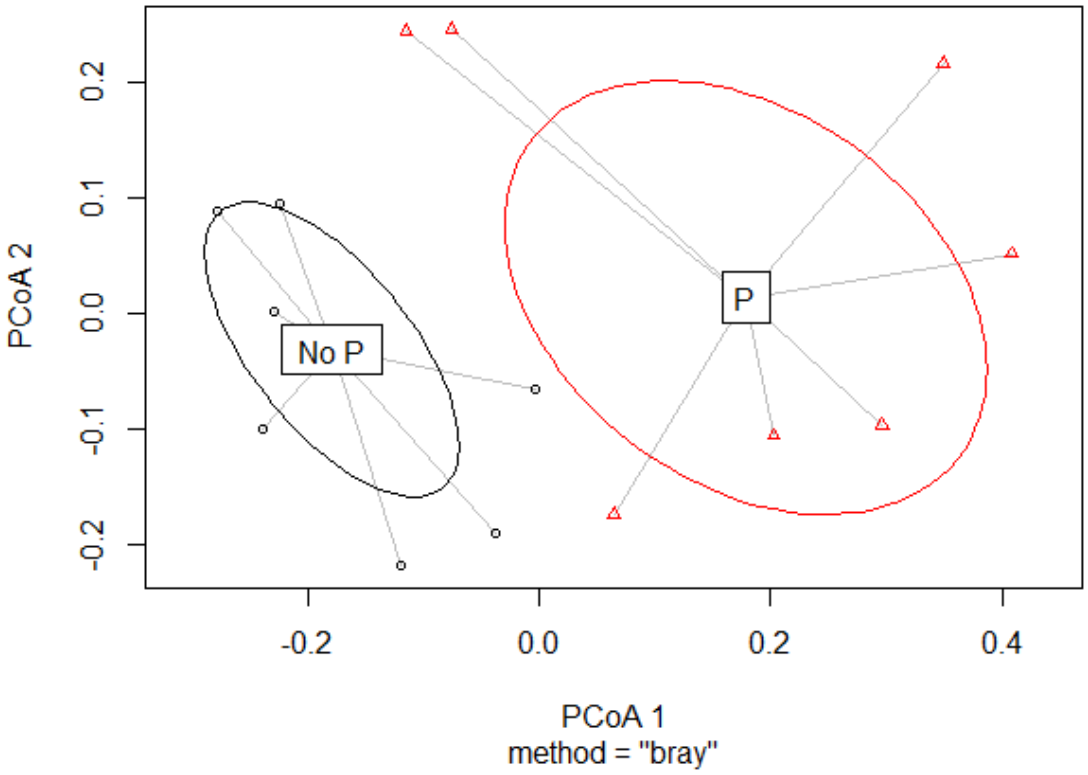

Figure 10: Principal Coordinates Analysis of fungal community data across all samples showing P-amended treatments (“P) and no added P (“No P”) Ellipses represent a 95 % confidence interval, separation between the two treatments is clearly apparent

Ecology

Both biostimulant treatment and P-amendments were found to affect Inverse Simpson index (Two-way ANOVA, $p = 0.008$ and $p = 0.002$ respectively). All biostimulants in both P- amended and non-P amended treatments had significantly lower inverse Simpson index values than the control (Table 5). P-amendments were also associated with significantly reduced Shannon index values (ANOVA, $p = 0.05$), and also significantly reduced overall fungal taxa count (ANOVA, $p = 0.01$) and OTU count (ANOVA, $p = 0.003$). Sterilising the biostimulant was associated with a significantly reduced total number of fungi in treated substrate (ANOVA $p = 0.05$).

Table 5: Differences in Inverse Simpson index values between biostimulant treatments and controls (no added P) which had a mean inverse Simpson Index of 21.21. Significance codes: 0.001 **, 0.01 *. Effects of biostimulant sterilisation on Inverse Simpson Index were not significant and are therefore not reported.

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

Figure 11 summarises mean abundance of the broad ecological groupings associated with the fungal taxa identified. Biostimulant treatment and P-amendments had a significant combined effect on Dark Septate endophyte abundance (Two-way ANOVA, $p = 0.04$), with significantly reduced abundance of DSEs compared to the controls (Tukey post hoc, $p < 0.05$). Saprophytic fungi abundance significantly increased with P-amendments (ANOVA, $p = 0.006$).

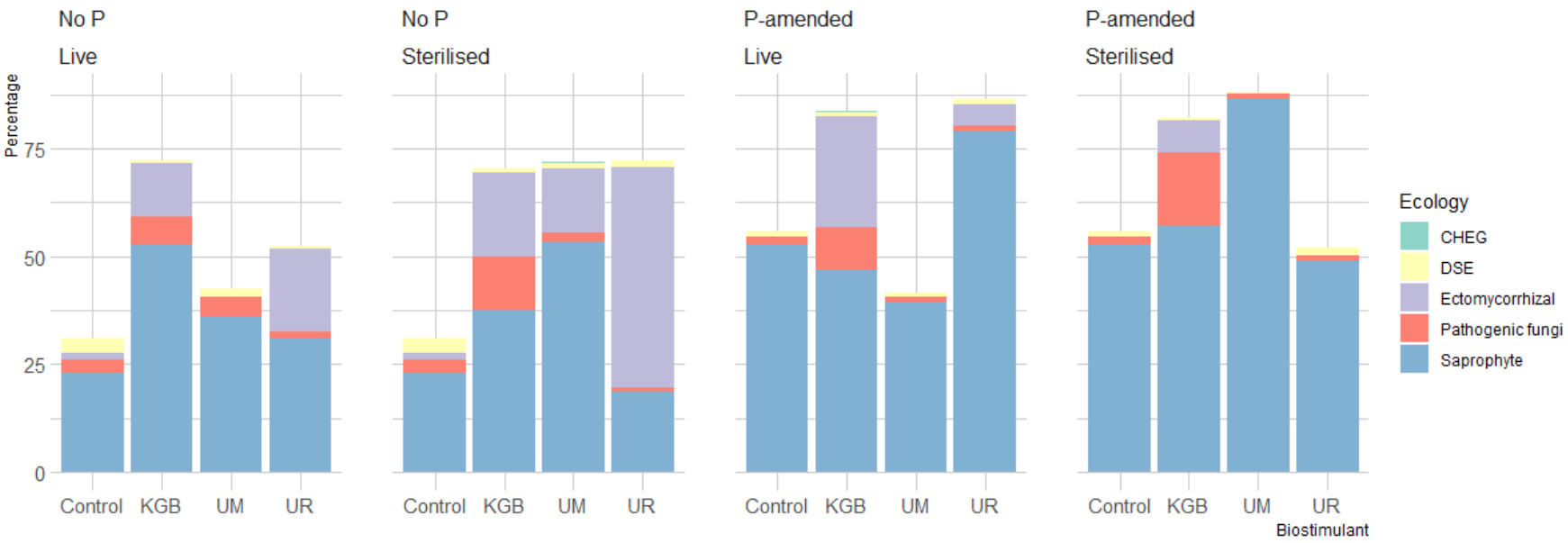

Figure 11: Stacked bar chart showing mean abundance of the five identified ecological groupings across the three live and sterilised biostimulant treatments and controls in P-amended and non-P amended substrate ("CHEG" = *Clavaria Hygrocybe Entoloma Geoglossum* (grassland fungi), "DSE" = Dark Septate Endophytes).

Discussion

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

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

In non-P amended treatments, live biostimulants generally performed less effectively than sterilised biostimulants in the regard of improved plant growth-promotion. Elemental analysis of the sterilised biostimulants confirmed that there were no significant changes to chemical composition after sterilising. This would suggest that in the experimental conditions in this study, the microorganisms introduced by the biostimulants negatively affected plant growth morphology (Berdeni *et al*., 2018; Romera *et al*., 2019). However, this does not mean that the

live biostimulants are unable to afford plant growth-promoting benefits in other soil conditions with different microbial community structures and different abiotic conditions (Awasthi, 2019; Santini *et al*., 2021). This is demonstrated by the live UR treatment plants outperforming other treatments in P-amended condition. These findings would support the general conclusion that no individual biostimulant will improve crop growth under all conditions. In this case, since the live UR biostimulant was associated with improved plant growth in P-amended conditions, it could be possible that microbial community shifts associated with improved plant growth-promotion are detectable. This would necessitate further research into changes in microbial community structure associated with beneficial changes in plant growth and morphology after biostimulant or nutrient amendments.

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

Usually, the parameters of roundness 2, isotropy and RMS will increase and decrease over time, which results from the cycle of leaf initiation and expansion (Pavicic *et al*., 2017). Rosettes will begin this cycle with an elliptical shape which develops into a more circular one

when the third and fourth leaves begin to develop. Since these leaves will continue to develop after the first two leaves have ceased to grow, an elliptical tendency will then be observed again in the second week. The repetition of this cycle explains the oscillatory nature of the circularity parameters, and may suggest the value of polynomial models or localised regression to visualise this data as opposed to other models (Pavicic *et al*., 2017).

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

In the P-amended soils displaying more growth from live biostimulants than sterilised ones, it is possible that microbial activity contributed to plant growth-promotion as a result of the treatment. Even if this was not the case that microorganisms were actively introduced through the biostimulant which aided plant growth, the treatment could still have stimulated microbial biomass growth through improved rhizosphere equitability to plant growth-promoting microorganisms. Further research would appear necessary to determine the specific factors that improve rhizosphere equitability for plant growth-promoting microorganisms.

The potting substrate used in this trial consisted of sterilised compost. This has a number of important implications. Firstly, this prevents any discussion from these findings as to the influence of priority effects, interactions between resident microbial communities in soil and introduced microbes from biostimulants, or metabolic niche overlap (Parnell *et al*., 2015; Kaminsky *et al*., 2019). Secondly, sterilised compost may result in greater nutrient availability

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

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

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

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

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

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

In this study, it was observed that live biostimulants had significantly higher taxa counts than sterilised counterparts, which suggests that the biostimulant treatments tested herein promote a proliferation of fungal taxa in the soils which continue to have effects at least two months after biostimulant application. This demonstrates that the method of biofixing microbes to the carrier substrate probably was to some degree successful in introducing and establishing

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

All biostimulants in both P- amended and non-P amended treatments had significantly lower Simpson diversity index values than the control, and P-amendments were also associated with reduced Shannon index values, the number of fungal taxa and OTU count. This could suggest that biostimulants can affect microbial community structure and soil equitability whether or not changes in crop growth are observable. Some live biostimulants (UR, containing DAP and zeolite) was associated with higher growth rates in elevated P, but its sterilised counterparts produced significantly greater growth rates in non-P treated soils. In non-P amended soils, UR treatments had the lowest mean inverse Simpson index, but it is not possible to determine whether this has any connection to improved equitability for plant growth-promoting species.

The findings of this research suggest that the products investigated can significantly affect fungal community structure whether or not they have been sterilised, which requires further investigation. *Saitozyma* abundance was significantly associated with KGB (BrassiKa) treatments, and in KGB-treated soils it remained relatively constant regardless of P-amendments or whether the biostimulant had been sterilised. Elemental analysis showed that KGB carrier composition consisted of 35 % carbon, whereas other biostimulants were all below 0.3 % C. These results may indicate that a carbon rhizosphere priming effect (Adamczyk *et al*., 2019) may be associated with *Saitozyma* abundance, but further research would be needed to corroborate this. Significant production of IAA (>1000 µg/g) is associated with the yeasts *Saitozyma* and *Solicoccozyma* genera (formerly *Cryptococcus*) as described in Streletskii *et al*. (2016). These effects are detectable in vitro, but field scale studies which account for this are lacking (Yurkov, 2018). Yurkov (2018) also describes *Saitozyma* diversity

as a possible indicator of soil equitability associated with well-drained acid soils, but as explained above, due to the experimental substrate lacking key biological similarities to agricultural soils, equitability indications cannot be made from these findings, which would require similar patterns to be observed in field studies under similar treatments (Lentendu *et al*., 2014; Hartmann *et al*., 2014).

A genus which displayed significant shifts in abundance associated with phosphorus was *Mortierella*. Other studies have documented reductions in *Mortierella* abundance after N application (Arnebrant, *et al*., 1990), the authors attributing the changes to difference in pH. A number of studies document the importance of pH as a determining variable of fungal community structure (Tedersoo *et al*., 2014), and since this study sought to restrict determining variables responsible for shifts in fungal community structure to P-amendments and biostimulant treatment, care was taken to ensure homogeneity of pH in the soil substrate. In contrast, Detheridge *et al*. (2016) documented positive correlations between *Mortierella* and soil N levels, and a negative correlation with soil P levels, although Detheridge et al. (2018) were examining changes in fungal community behaviour in field conditions. In the present study, it was observed that in P-amended treatments, the genus *Mortierella* constituted the majority of fungal taxa observed in the live UR-treated soil, and was also the most abundant genus in the soil treated with sterilised UM. Both treatments produced the largest growth curves in their respective conditions, which would suggest a possible role of *Mortierella* in improved P-use efficiency. Detheridge *et al*. (2016) propose that *Mortierella* may be actively involved in P mobilisation from inorganic sources by organic acid secretion, which can chelate bound cations. Neither *Mortierella* nor *Saitozyma* are likely to have endophytic/mycorrhizal modes of action, as Nallanchakravarthula *et al*. (2014) were able to demonstrate by showing that neither genus were present in root tissue but were the most abundant taxa in the rhizosphere. In pot trials similar to this study, *Mortierella* has been associated with increased plant P uptake, and may act synergistically with arbuscular mycorrhizals (Osorio and Habte, 2013). However in this study, there were no obvious associations between *Mortierella* relative abundance and improved crop P uptake, other than that the genus was more abundant in soils which had higher plant available P. In any case, the behaviour of *Mortierella* in this study was not being studied in a representative agricultural soil (Lentendu *et al*., 2014; Hartmann *et al*., 2014), and therefore more research in field environments will be necessary to quantify whether the possible mode of action described above can influence crop yield.

In this study, a combined effect of the UM biostimulant and P-amendments were found to increase *Trichoderma* abundance. This species is a well-documented plant growth-promotor, and is a common species used in biostimulants. Stewart and Hill (2014) describe its effects in detail, which include increased root and shoot biomass as well as improved morphology, which

are likely a result of improved hormone balance with respect to IAA, ethylene and gibberellic acid. An uncertainty often reported with biostimulants relates to inconsistency and viability of batches, and this study has demonstrated viability of *Trichoderma* in treated soils at least two months post-inoculation (sterilising the biostimulant resulted in only trace abundance of *Trichoderma*). Again, these findings do not account for the competition for nutrients in agricultural soil that introduced microbes will face (Romera *et al*., 2019), so further work is necessary to determine whether hormone-balancing effects or pathogen suppression effects (often attributed to *Trichoderma;* Berdeni *et al*. (2018)) are observable months after inoculation, and therefore this kind field experiments will be necessary to corroborate these findings. It was not possible within this study to discern if the increased abundance of *Trichoderma* was of native or applied origin, as *Trichoderma* can be readily found in the environment and it is possible that come airborne contamination through the facility ventilation system could have introduced *Trichoderma* spores to the experimental substrate (Symbio, 2019). Future methodologies may be able to address this by culturing *Trichoderma* isolated from the biostimulant and contrasting genotypes with *Trichoderma* sequenced from the treated soils (Mukherjee *et al*., 2013), or experimental procedures could include operating in even more sterile environments with purified ventilation to prevent airborne spore ingress. This would of course be even less representative of agricultural soils and would restrict the degree to which interpretation could be made relating to purported microbial modes of action, but it would clarify the extent to which cross-contamination could have led to the establishment of supposedly plant growth-promoting microbes that have been claimed to have been introduced to soils by biostimulants (Hart et al., 2018). In this study, the background microbial count and composition of the microbial communities living on the seeds were not examined, which could include species which later established in the experimental substrate. Attempts were made to investigate the microbial composition of the biostimulants before they were introduced to the soil to confirm the manufacturer's information on species composition presented in Table 1. Unfortunately, neither ordinary molecular diagnostic nor PowerSoil extraction methods obtained results, and separate work should be undertaken to verify the manufacturer supplied information on species composition, which could be misleading for various reasons (Hart et al., 2018 Kaminsky et al., 2019).

Sterilised biostimulants in non-P amended soils had a significantly higher abundance of ectomycorrhizal fungal taxa, and this treatment pairing was associated with the largest overall plant area during the growing period, although not the highest levels of crop P uptake. In particular, sterilised UR treatments were associated with the largest number of ectomycorrhizal fungi in non-P treated soils (mainly consisting of the genus *Sphaerosporella*). As the biostimulants were sterilised, they could not directly have introduced ectomycorrhizal

fungi to the rhizosphere but they may have contributed to improved equitability for ectomycorrhizal species, possibly through zeolite-mediated effects which have elsewhere been documented to improve P-use efficiency in plant tissues (Ahmed *et al*., 2010). Ectomycorrhizal fungi usually form symbioses with woody plants, but recently Detheridge *et al.* (2016) have suggested that some ectomycorrhizal fungi may form mutualisms or endophytic associations with non-woody host species in agricultural contexts. The present study supports this finding and would suggest that it should be subject to further research, given that the experimental substrate was not representative of agricultural soils, as explained earlier.

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

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

4.6. Conclusion

The method used in this study was able to document differences in growth rates and a comprehensive range of morphological parameters of an *Arabidopsis* crop treated with different biostimulant and phosphorus forms. This provided detailed insights into the responses of the experimental crop to the effects of the treatments used. The study suggested

889 that under elevated P, some of the live biostimulants examined herein were more efficacious
890 in improving plant growth and morphology than under reduced P, but that treatments were not
891 actually associated with improved crop P uptake. The findings also suggest that some
892 supposedly plant growth-promoting microbial cultures actually stimulate negative responses
893 from the plant in certain conditions, indicating a probable metabolic cost to the plant host. This
894 requires further research to examine what shifts in community structure occur after
895 biostimulant treatments have been applied, and requires contextualising in representative
896 agricultural soils.

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898 **4.7. References**

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