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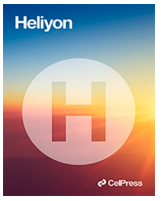

Research article

Humate application alters microbiota–mineral interactions and assists in pasture dieback recovery

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ABSTRACT

Pasture dieback is a rapidly expanding decaying pasture syndrome that affects millions of hectares of agricultural land in Queensland, Australia, making it useless for the cattle industry and decimating farmers' income and welfare. Since the syndrome was first identified in the early 1990s, farmers and agronomists have tried various methods for pasture recovery, including slashing, burning, ploughing and resowing grass, fertilising, destocking, and overstocking. In most cases, after a minimal initial improvement, the grass reverts to dieback within a few weeks. Here, we present an application of potassium humate, a well-known plant growth stimulator, as a possible long-term recovery option. Humate was applied once at the rate of 12 ml per m^2. Humate application did not alter the alpha or beta diversity of soil bacterial communities, nor did it change the mineral profile in the soil. However, humate application altered soil microbiota–mineral temporal interactions and introduced subtle changes in the microbial community that could assist pasture recovery. A single humate application increased paddock plant biomass significantly up to 20 weeks post-application. Eleven months after the single application, the paddock was grazed to the ground by the cattle just before the rainfall season. After pasture regrowth, the humate-treated plots significantly improved root morphometric indicators for both grass and dicots and increased the ratio of grass/weeds by 27.6% compared to the water-treated control. While this treatment will not resolve the dieback syndrome, our results invite more research to optimise the use of humate for maximum economic benefit in paddock use under pasture dieback syndrome conditions.

1. Introduction

Beef is an essential source of nutritional protein for many people around the world, with a global cattle herd size of approximately one billion heads [1] estimated to provide 45% of the global protein supply for human consumption [2]. The Australian cattle herd was 24.7 million head in 2019, with Queensland cattle accounting for 45% of the Australian herd [3].

Pasture is essential for cattle production as the base of sustainable farming, providing the animal with all the nutritional requirements needed to grow and produce good quality meat, including protein, energy and micronutrients like vitamins and minerals. Pasture-based cattle production depends significantly on the quality and availability of high nutritional value plants [4] the animal can consume. A pasture which is diverse in species will deliver a more balanced, suitable quality and abundance of nutrients to the animal

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[5]. Pasture production is highly dependent on soil health and management practices [6].

Pasture dieback (PD) is a decaying pasture syndrome of yet unknown causes affecting grass production across multiple soil types and landscapes, reducing its carrying capacity and impacting livestock production [7]. Grazing avoidance in these areas was observed in cattle, however, the effect on animal health is unknown. PD appeared in Queensland around 20 years ago, affecting different cultivars of buffelgrass (*Cenchrus ciliaris*) [8]. The most affected buffelgrass cultivar was "American" (USA buffelgrass), while the Biloela cultivar was considered by many as the most resistant to dieback [8]. Currently, dieback is reported to affect many grass species, including several Australian natives and introduced species [9]. Farmers report many more species affected, including Angleton grass (*Dichanthium aristatum*), couch grass (*Cynodon dactylon*), Kikuyu grass (*Pennisetum clandestinum*), Queensland blue couch grass (*Digitaria didactyla*), nutgrass (*Cyperus rotundus*), and even some legumes. This indicates that PD can affect a range of species [10].

In 2016 the Queensland Government Department of Agriculture and Fisheries (DAF) reported that the dieback affected approximately 35,000 ha of Queensland pasture. At present, the near-exact affected surface area is unknown, but it was estimated to be in millions of hectares of pastures in QLD and northern NSW [7]. Dieback often appears as patches at first about 5 m^2 in diameter [11], then spreads and merges into larger areas, rapidly affecting the whole paddock and, in some instances, the whole farm.

PD appears first in older leaves, as reddening or yellowing of the tip and spreading to the base of the ligule, before moving to the rest of the plant. Roots become stunted and underdeveloped. Under microscopic observation, conduction vessels (xylem) appear obstructed, and lesions on roots were observed with cellular damage [8]. Many studies are being conducted on PD with different theories about the possible causes. Some of the theories implicate damaged soil microbiota, increased pathogens, eroded soils, reduced organic matter, poor soil management, introduced species (weeds as well as insects), deforestation, loss of diversity, drought stress and a combination of factors, including insect infestation with mealybugs [11] or ground pearls [10]. Despite numerous suspected factors, there are no peer-reviewed publications, conclusive results or enough data to claim causality, and the principal source of PD remains unknown. Some improvements were recently reported using a single application of sea minerals [12] and a product containing commercial plant growth stimulant tri-sodium salt of trimercapto-S-triazine (TMT) and potassium humate as active ingredients [13]. Both treatments resulted in various soil microbiome and pasture yield improvements.

One of the priorities of sustainable agriculture is to seek ecologically friendly ways to improve plant productivity and quality [14]. Potassium humate and humate substances are natural organic components of soil humus [15]. They can originate from soil, peat, plants and coals [16]. Fulvic and humic acids are among the most biologically active and complex organic compounds [17]. They are known to have many impacts on soil, such as chelating of metals and improving soil's physical properties [15]. They can improve nutrient availability and enhance chemical and biological soil processes [18]. They also positively affect plant roots, especially in root hair initiation and lateral root growth and density which are involved in nutrient uptake [17]. Humic acids help plants cope with stress conditions like salinity, extreme temperature and pH [18].

All these improvements could be related to the soil microbial community alterations, as studies show that humic acid can increase plant defence by stimulating plant-associated bacterial communities to protect against pathogens. Humic acids can recruit bacterial species that can suppress pathogens. Jin et al. [19], found that humic acids can reshape soil microbial profile and function and affect microbial reproduction. Humic acids stimulate bacterial species from phylum *Chloroflexi*, *Acidobacteria* and *Actinobacteria* that break down organic matter, alter available soil minerals and increase plant access to nutrients [19].

The use of humic acids as organic fertilisers has increased lately as its application reduces the need for inorganic fertilisers while improving soil properties such as drainage, water retention capacity, deactivation of toxic metals [20], aeration, aggregation stability and facilitation of micronutrient availability [21]. Foliar application of humic acids has a growth-promoting effect attributed to hormone-like influence. Humic acids accelerate cell division, increase plant cell permeability, activate photosynthesis, improve nutrient uptake, and decrease the uptake of toxic elements [22].

Humic acids are commonly used for restoring degraded soils, with longer-lasting effects than inorganic compounds. Minimal concentrations can enhance plant growth and quality and soil nutrients in plant-available form. It is established that the use of humic acids can increase the availability of Ca, P, K, N, Mg, Mn, Cu and S [17]. Interaction with soil microbial communities can stimulate or inhibit plant growth, making plants more resistant to stress and increasing yield and nutritional value [23].

Humic acids used as fertiliser often contain 2.8% phosphorus, 10% potassium, 5% nitrogen and micronutrients like copper, zinc, molybdenum, and cobalt. The importance of K^+ in photosynthesis increases enzyme activity, boosting the synthesis of carbohydrates, proteins and fats, enabling the plant to resist pests and diseases [22]. The application of humic acids has a stimulant effect on cytokinin, gibberellin and auxin substances [18]. The application of high humic acid concentrations showed depressing effects on plant growth, and therefore it should not exceed a rate of 20 L/ha when applied together with a K seed dressing [24].

In this study, we used potassium humate (Hum) application on the PD-affected soil to investigate its ability for remediation and possible improvements in soil microbial diversity and community structure.

2. Materials and methods

2.1. Farm description and location

The experiment was conducted on a farm located in Garnant, Queensland. The property is 1500 ha and carries 800 head of *Bos indicus* crossbred beef cattle. In 2017 the grass started to die off, and in 2021 the farm had around 100 ha of PD-affected grass. The paddock used in this trial was 38 ha of brown dermosol soil pastured land. From 2017 to the time of the experiment (June 2021), the region has experienced severe drought. According to farm records, the pasture species profile before 2017 included: black spear grass

(*Heteropogon contortus*), buffelgrass (*Cenchrus ciliaris*), forest blue grass (*Bothriochloa bladhii* ssp *glabra),* Queensland Bluegrass (*Dichanthium sericeum*), Green Panic (*Megathyrsus maximus*), Indian Couch (*Bothriochloa pertusa*), kangaroo grass (*Themeda triandra*), Seca stylo (*Stylosanthes scabra*), and native legumes and was considered very productive. By 2019 all grass was affected by PD, and there was no new growth in the affected area. In 2020 the paddock became abundant in phasey bean (*Macroptilium lathyroides*), a naturalised legume. Other dicot species seemed to recover, but very little grass was observed. The paddock seemed to be rapidly transitioning through various species. In 2021 this paddock showed signs of dicot recovery, dominated by common weeds. The species growing in the PD-affected area were not optimal for cattle nutrition, and they included Indian couch (*Bothriochloa pertusa*), wild sage (*Salvia verbenaca* L), some woody plants such as currant bush (*Carissa ovata* and *Carissa lanceolata*) and small annual herbs, with many areas showing minimal or no plant growth. Cattle only grazed Indian couch and avoided grazing any of the other species, thus reducing the pasture carrying capacity.

Although we intended to include the plots with the healthy, PD-unaffected soil, in all farms we considered this was not an option because the whole paddocks were either fully affected, or the patches of grass in the paddock that appeared healthy were in the pre-clinical stage of the dieback syndrome. Therefore, both control (untreated) and potassium humate (Hum) treated plots were set on the PD-affected paddock.

3. Methods

The experiment used a randomised complete block design with three replicates for control (Ctr) and potassium humate treated plots (Hum) each. The area of each experimental plot was 5 m by 5 m, separated by a 2 m buffer area between plots to avoid drift contamination while spraying. The Hum marketed by Humic Solutions, Australia, was diluted using untreated water at a ratio of 6 ml/L and applied once at a rate of 2 L of diluted product per square meter using a backpack garden sprayer to simulate farming spraying equipment. Control plots were sprayed with the same amount of water (2 L water per square meter).

Soil samples were collected with the core soil collection method [25], using a T-bar core sampler at 15 cm depth to include rhizhosphere soil. Samples were taken from all plots before applying Hum and weekly after the spraying for six weeks, then fortnightly until week 20; these were used for DNA extraction and microbiota assessment. Samples were kept on ice during transport and stored in a −80 °C freezer until used for laboratory analysis. Originally, the first sampling of dry matter, soil parameters and plant morphology were planned for 12 weeks post-application, but plots and, consequently, plant measurements were disturbed due to the damage of grass by kangaroos. Plots were then recovered, and samples of dry matter were collected at 20 weeks. Plant measurements were collected again at eleven months (48 weeks after Hum application) after cattle grazing of vegetation to the ground and a high rainfall event to evaluate long-term effects. This time, separating the plant samples into monocotyledons, dicotyledons, and litter was possible.

Plant samples were taken before spraying Hum, and at week 20 and week 48 of the trial, using a 50 × 50 cm quadrat thrown at random twice per plot, cutting and collecting all plant material using scissors as close to the ground as possible. Separation of vegetation into species at weeks 0 and 20 was impossible as PD-affected grass disintegrated on contact. The samples were dried using an oven at 60 °C for 72 h and then weighed and recorded in a table to compare and calculate dry matter per hectare.

Plant samples for morphological studies were collected at week 20, and week 48 by choosing representative grass and weed (wild sage) specimens per plot (same species on each plot for both 20-week and 48-week sampling), collecting whole plants with the root system intact, and keeping them on dry ice until measurements were conducted. Root width was measured using callipers, while plant height, root length, and size of leaves were measured using a ruler. Seed heads, the number of branches/tillers, and the total number of roots were also counted.

Soil minerals were assessed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP – OES). This instrument can measure over 20 different soil minerals in 4 min [26]. For this experiment, the soil samples analytics were outsourced to the University of Western Australia's Earth and Environment Analysis Laboratory (EEAL) located within the School of Agriculture and Environment to obtain mineral content (including heavy metals). All minerals in the detection range of the methodology were measured, including total Carbon/Nitrogen, Al, B, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Zn, pH, and Electric Conductivity.

Soil microbiota molecular analysis was done by directly extracting soil DNA using a DNA soil kit (DNeasy PowerSoil Pro Kit) and following the manufacturer's recommended protocols. The soil sample was processed to extract a crude lysate, followed by purification steps to remove compounds like fulvic and humates that contaminate the sample and obstruct subsequent PCR sequencing [27]. After DNA extraction, the 16S rRNA gene sequencing library was prepared by amplifying the V3–V4 hypervariable region of the 16S rRNA gene using dual indexing, variable spacer primers; with the forward 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). The sequencing was outsourced to Azenta Life Sciences (China) and performed using a 2 × 300 paired-end sequencing and Illumina MiSeq system using all Illumina recommended kits and protocols.

Data analysis was done using Qiime2 software, with denoising and chimera checking performed by Dada2. The ASV level data was clustered to an OTU level using 98% similarity. The samples with less than 1000 sequences were removed from the analysis leaving a total of 57 samples. An OTU level data was normalised using Hellinger transformation and further analysed using a range of R packages, including phyloseq, vegan and microecco. Primer-e v7 (https://www.primer-e.com/our-software/primer-version-7/) was used to further explore and present the data. Plant data, including dry matter and plant morphometrics, was analysed and presented using GraphPad Prism v9 (https://www.graphpad.com/scientific-software/prism/). Raw sequences are publicly available on Sequence Read Archive (SRA) database under accession number PRJNA887675.

4. Results

4.1. Overall community structure

The relative abundance at the phylum level presented in Fig. S1 of Supplementary File1 displays a clear dominance of *Actinobacteriota* (corresponding at 61% of sequences), followed by *Proteobacteria* (10%), *Chloroflexi* (7%), *Firmicutes* (6.15%), *Acidobacteriota* (6.03%), *Gemmatimodota* (2.5%), *Myxococcotta* (2.4%), *Verrucomicrobiota* (1.7%), and with less than 1% of sequences assigned to *Methylomirabilota, Entotheonellaeota, Planctomycetota, Nitrospirota, RCP2-54 and Bacteroidota.* The *Archaea* from the phyla *Crerchaeota* and *Thermoplasmatota* were also detected (0.10 and 0.004%, respectively). Fig. 1 shows the relative abundance of the top twelve genera; the most abundant genera were *Rubrobacter, Solirubrobacterales 67-14, Un. Gaiellales, Bacillus, Un. Xanthobacteraceae, Conexibater, Gaiella, Un. Micromonosporaceae, Bacillales, Solilubrobacter, Actinobacteriota MB-A2-108, Un. Solilubrobacterales*.

4.2. Alpha and beta diversity

Alpha diversity indicated by Simpson, Richness, and Pielou's evenness is shown in Fig. S2 of Supplementary File 1. The alpha diversity indicators over the 20 weeks show that potassium humate (Hum) did not change the microbial diversity.

In the investigation of the community structure, we used three distance measures: Bray Curtis, Weighted and Unweighted Unifrac distance. We first inspected the sample-to-sample distance within each group to investigate the uniformity of samples within each group using Anosim multivariate analysis (Supplementary Fig. S3). Analysis using weighted distances (Bray Curtis and Weighted Unifrac), that consider the taxa abundance, revealed a significant difference in within-group sample-to-sample distance between the Ctr (control) and Hum (potassium humate) treated soil. Alternatively, Unweighted Unifrac, which looks only at the presence and absence of taxa, showed that these differences within each group were not significant. This indicates that the higher sample-to-sample difference in the Hum group is related to a change in abundance rather than the presence or absence of taxa (Fig. S3).

We used Primer-7e and Primer PERMANOVA plugin to investigate community structure using mixed design Permanova with "Week" (as numeric) and "Group" (2 levels Ctr and Hum) as crossed fixed effects and "Plot number" as a random effect nested within "Group" as recommended for the longitudinal sampling of multiple plots for treatment and control (http://updates.primer-e.com/primer7/manuals/PERMANOVA+_manual.pdf). Using either Bray Curtis, Weighted or Unweighted Unifrac distance, "Week" (time) was always significantly affecting the microbial community ($P < 0.0001$; $P = 0.0008$, $P < 0.0001$, respectively). "Group" was not significantly altering microbial community using either distance ($P = 0.7048$, $P = 0.36$ and $P = 0.7989$, respectively). Neither of the interactions was significant by either distance. This indicated that the microbial community was fluctuating weekly and that these major weekly fluctuations were not due to the use of the Hum but more likely due to environmental effects that were affecting all plots at once (rainfall, heat stress etc.). If so, it is likely that these climate-related effects were overpowering any direct influence of Hum application. However, Hum treated and untreated plots responded to environmental changes very differently (Fig. 2), and the

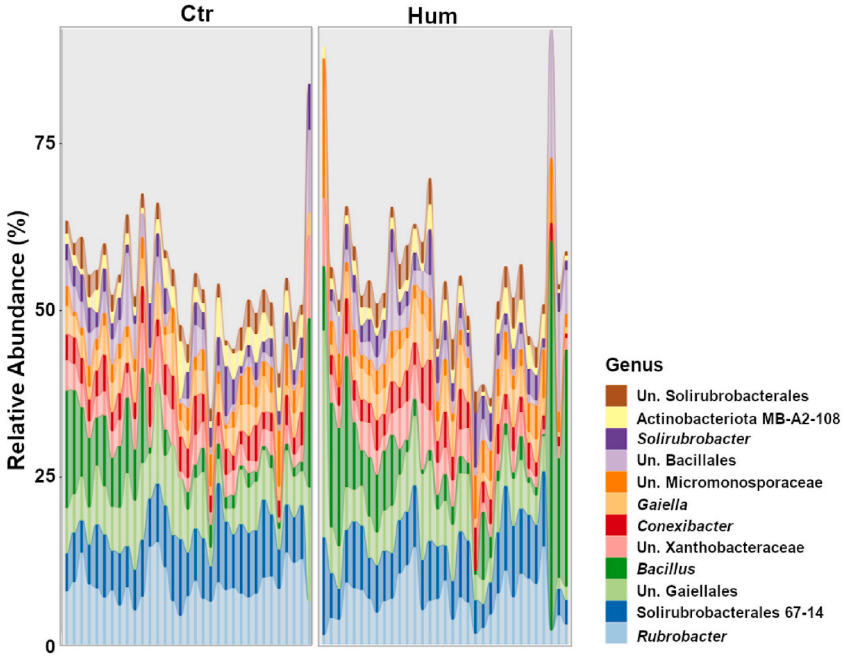

Fig. 1. Microbiota community structure at a genus level showing top twelve genera in control (Ctr) and potassium humate (Hum) treated soil over time.

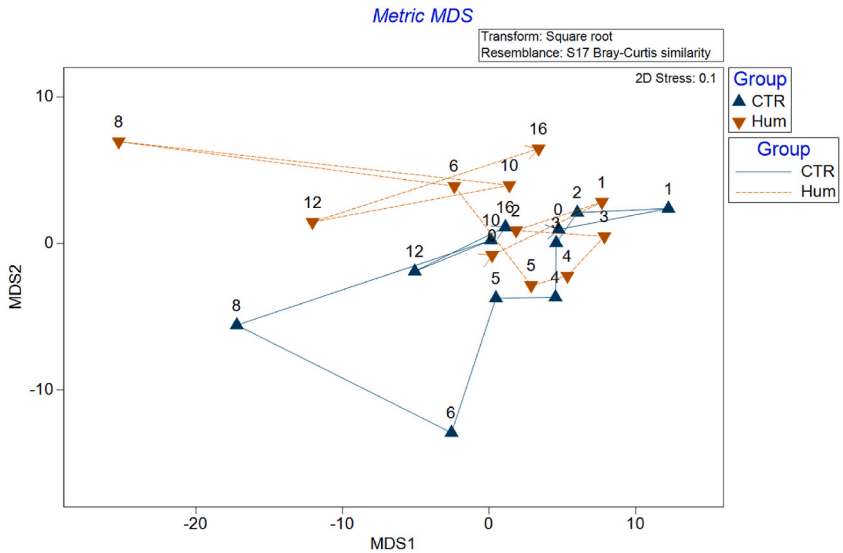

Fig. 2. MDS plot of weekly distances for control (Ctr), and potassium humate (Hum) applied once presenting community shifts driven by environmental factors (rain event). The major rain events after the prolonged drought occurred in week 5 and week 7, with no further rain after week 8.

differences in microbial communities became apparent after the rain and disappeared after the soil reverted to dry.

To further investigate and visualise these weekly alterations and match them to the environmental conditions, we calculated distances among centroids for each combination of "Group" and "Week" and observed weekly microbial community shifts for Ctr and Hum separately using Primer 7e (Fig. 2). The MDS plot shows a minimal distance between Ctr and Hum in weeks 0, 1,2,3,4 and 5. The Ctr and Hum microbial communities started to diverge from week 6 to week 16, with Ctr returning to the original structure after week eight and Hum remaining relatively distinct from the original community (Fig. 2).

Table 1
Simper analysis of the mixed effect Permanova design. The 27 genera presented in the table account for 50.29% of the variation between Ctr and Hum.

Genus	Individual Contribution (%)	Cumulative Contribution (%)
Gaiellales uncultured	4.22	4.22
Solirubrobacterales 6714	3.79	8
Bacillus	3.56	11.56
Rubrobacter	3.04	14.6
Micromonosporaceae unclassified	2.82	17.42
Actinobacteriota MB A2 108	2.59	20.01
Gaiella	2.56	22.57
Acidothermus	2.47	25.04
Thermomicrobiales JG30 KF CM45	1.86	26.91
Chloroflexi TK10	1.82	28.73
Gaiellales unclassified	1.71	30.44
Solirubrobacter	1.55	31.98
Candidatus udaeobacter	1.51	33.49
Solirubrobacterales unclassified	1.48	34.97
Conexibacter	1.42	36.39
Elsterales uncultured	1.4	37.79
Micromonospora	1.34	39.13
Acidimicrobiia IMCC26256	1.33	40.45
Geodermatophilus	1.26	41.71
Bacillales unclassified	1.22	42.93
Gemmatimonadaceae uncultured	1.19	44.12
Jatrophihabitans	1.11	45.24
Vicinamibacterales uncultured	1.09	46.32
Pseudonocardia	1	47.32
Planosporangium	0.99	48.32
Streptomyces	0.99	49.31
Xanthobacteraceae unclassified	0.98	50.29

4.3. Univariate analysis

The lack of significant community alterations via multivariate Permanova indicates only a lack of major overall community alterations. To investigate targeted alterations, we used Simper analysis on mixed-effect Permanova design (Primer-7e, Table 1) to identify the taxa that are contributing to between-group dissimilarity. *Gaiellales* is the genus most contributing to the difference between control (Ctr) and potassium humate (Hum) by 4%, and *Solilubrobacterales*, *Bacillus*, *Rubrobacter* and *Micromonosporaceae* cumulatively contribute over 15% to the difference between Ctr and Hum.

LefSe acts as a biomarker discovery tool for all taxonomic levels. Intriguingly, the Hum group is enriched with *Firmicutes*. In contrast, the control group is enriched with *Actinobacteriota* (Fig. 3). Other differential taxa were visualised in Fig. 3, where *Rubrobacteria* in all taxonomic levels behave as a marker for control, as well as *Chloroflexi, Solilubrobacteriales, Actinibacteriota* and *Actinomycetales*. Biomarkers for Hum are *Microsporales* and *Bacilli* in all taxa levels, as well as *Gailellales, Thermophilia* and *Firmicutes*.

4.3.1. Correlation analysis

The correlation of taxa with time on Hellinger normalised genus-level data was performed separately in control and Hum-treated plots (Table 2) to observe the possible alterations in the temporal development of the soils treated with Hum. The genera significantly negatively correlated with time (reducing) in control but not in Hum plots are *Marmoricola, Actinoplanes, Asanoa, BIrii41, Dactylosporangium, Ellin6067, Actinomadura, Angustibacter, Cryptosporangium, Dongia, G12-WMSP1, Haliangium, JG30-KF-CM66, Nocardioides, Reyranella, Saccharopolyspora, Skermanella, Streptomyces, Vicinamibacteraceae* and *mle1-7*. The most significantly negatively correlated with time in the Hum-treated soil were *KD4-96, Pseudonocardia, AD3, Gaiella, JG30-KF-CM45, Anaeromyxobacter, Cohnella, Gemmatimonas, Gitt-GS-136, Rhizobiales, Rubrobacter* and *bacteria p 25* and these remained not significantly affected by time in control.

4.3.2. Mineral profile

Soil chemical parameters in both treatments were measured at week 0 and week 12. Fig. S4 of Supplementary File 1 shows no statistically significant difference in mineral concentration between the treatments, although there were minor changes in the concentrations over the time from week 0 to week 12, none of them statistically or biologically significant.

The interactions of minerals and other soil parameters with the microbiota were investigated using microeco R package. To visualise environmental and biota interactions and find which minerals influence microbial communities, we used distance-based redundancy analysis (dbRDA) plots generated using Weighted and Unweighted Unifrac distance (Fig. S5 of Supplementary File 1) and RDA plots at genus and phylum levels (Fig. S6 of Supplementary File 1).

We then analysed correlations between genus and phylum level taxa with the concentration of specific soil chemical parameters. There was a clear difference in mineral association with taxa between Hum-treated and Ctr plots (Fig. 4). The top 10 phyla are shown in Fig. S7 of Supplementary File 1. In the samples collected from Hum plots, there were hardly any interactions with measured soil parameters (only three significant), while on Ctr plots, we observed 14 significant soil minerals/parameters – phylum correlations. Similarly, at the genus level, the correlated genera are shown in Fig. 4 heatmap, where high differences in taxa mineral correlations between treatments were even more prominent. This is in agreement with the well-established interaction of humic acids with soil minerals and nutrient decomposition and bioavailability discussed in the introduction.

Finally, we were able to observe the influence of minerals on alpha diversity indices (Fig. S8 of Supplementary File 1) where the

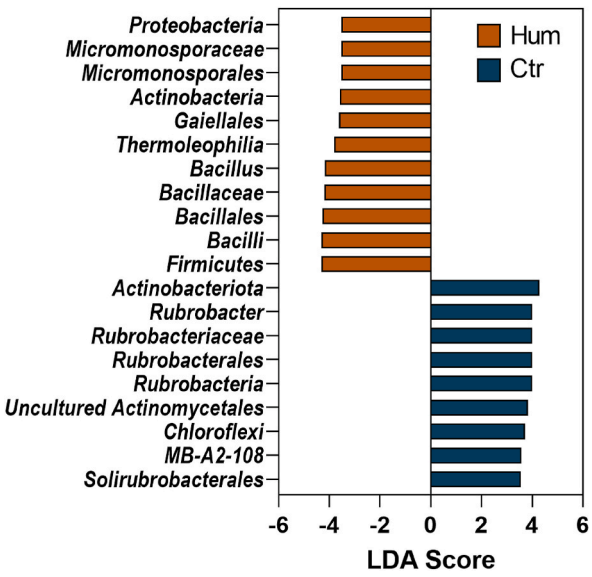

Fig. 3. LefSe plot at all taxonomic levels.

Table 2
Correlation with time on Hellinger normalised genus-level data, separately in control and Hum-treated plots. Genera correlated with time only in control are in blue font, and only in Hum are in orange font. Rho: spearman rank correlation coefficient.

	Ctr			Hum		
Genus	rho	P-value	Significance	rho	P-value	Significance
67–14	−0.63402	7.45E-05	***	−0.44784	0.008963	**
AD3	0.02461	0.891867		−0.50633	0.002642	**
Actinomadura	−0.39761	0.021939	*	−0.09155	0.612377	
Actinoplanes	−0.49775	0.003204	**	−0.21546	0.228508	
Anaeromyxobacter	−0.15774	0.380656		−0.34699	0.047885	*
Angustibacter	−0.35156	0.044829	*	0.227178	0.20358	
Asanoa	−0.45562	0.00771	**	0.016059	0.929319	
BIrii41	−0.50664	0.002624	**	0.01732	0.923787	
Cohnella	0.066804	0.711847		−0.35268	0.044108	*
Cryptosporangium	−0.41453	0.016463	*	−0.11181	0.535585	
Dactylosporangium	−0.48942	0.003845	**	−0.33673	0.055346	
Dongia	−0.39744	0.022	*	−0.23842	0.181502	
Ellin6067	−0.46402	0.006526	**	−0.0213	0.906351	
G12-WMSP1	−0.39756	0.021957	*	−0.08515	0.637543	
Gaiella	−0.33613	0.055807		−0.46007	0.007062	**
Gemmatimonas	−0.0495	0.784438		−0.40727	0.018653	*
Gitt-GS-136	−0.10438	0.563192		−0.41691	0.015793	*
Haliangium	−0.38985	0.024914	*	−0.17958	0.317306	
IMCC26256	−0.45526	0.007763	**	−0.37298	0.03253	*
JG30-KF-CM45	−0.29927	0.090665		−0.48421	0.0043	**
JG30-KF-CM66	−0.34634	0.048331	*	−0.059	0.744312	
KD4-96	−0.33389	0.057561		−0.58895	0.000311	***
MB-A2-108	−0.47199	0.005551	**	−0.54299	0.001094	**
Marmoricola	−0.68787	9.72E-06	***	0.078682	0.663387	
Micromonospora	−0.37139	0.033341	*	−0.38828	0.025552	*
Nocardioides	−0.42238	0.014339	*	−0.28932	0.10246	
Pseudonocardia	−0.10772	0.550723		−0.62892	8.85E-05	***
Pyxidicoccus	−0.36288	0.037935	*	−0.36249	0.038158	*
Reyranella	−0.40455	0.019535	*	−0.30144	0.088231	
Rhizobiales	−0.06456	0.721137		−0.36249	0.038158	*
Rubrobacter	0.077491	0.668189		−0.39148	0.024262	*
Saccharopolyspora	−0.36561	0.036406	*	0.01614	0.928965	
Skermanella	−0.40148	0.02057	*	NA	NA	NA
Streptomyces	−0.41919	0.015172	*	−0.24759	0.164773	
Vicinamibacteraceae	−0.37926	0.029499	*	0.084805	0.638906	
bacteria p 25	−0.1677	0.350892		−0.39315	0.023612	*
mle1-7	−0.47416	0.005308	**	0.13211	0.463636	

heatmap was split into two clusters of minerals: K, Pb, N, C, Al, S, EC and Fe reproducibly marginally reducing, and Cr, Mo and P marginally increasing richness and diversity measures. Although neither of these interactions was significant, Fe displayed clear negative and Cr positive correlations with the diversity.

4.3.3. Dry matter

All the changes in microbiota are reflected in plants by significantly increased dry matter in the Hum treatment compared to control at 20 weeks (Fig. 5 A), as well as the dry matter before application. At eleven months after treatment, there was an increase in monocotyledons (Fig. 5 B, C), a decrease in dicotyledons (Fig. 5 D), and slight change in litter (Fig. 5 E) in potassium humate (Hum) treated plots; however, not statistically significant.

4.3.4. Plant morphometrics

Plant morphometrics was taken at week 20 (Fig. 6), and after the major rain event at eleven months (week 48) (Fig. 7) for grass and weed (wild sage) that were representative of the whole plot. Although there were no statistically significant differences between the control and Hum-treated group, plant height, the number of tillers and the total number of roots of the grass in the Hum group were increased compared to the control. The sage showed a marginal increment in the number of branches and a number of seed heads in the Hum group.

Plant morphometrics collected post rainfall, eleven months after a single Hum application (Fig. 7), showed a statistically significant increase in the total number of roots in the grass, as well as a minor increment in plant height compared to the control. In the sage, there was a statistically significant increase in younger leaf length as well as the total number of roots. Therefore, even eleven months post-application Hum improved roots in both grass and sage.

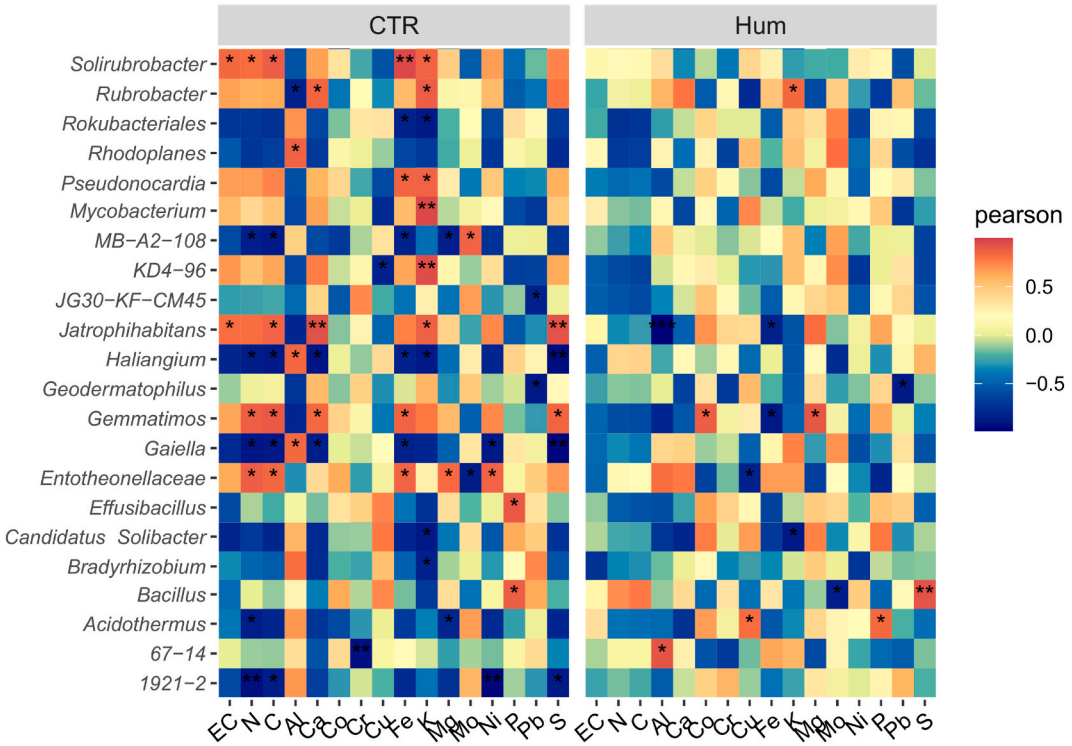

Fig. 4. Heatmap of significant mineral correlations with soil genera. Only significant correlations are shown on the map.

5. Discussion

Soil microbial communities are responsible for nutrient transformation and release, as well as biodegrading organic material and pest and disease control, among many other benefits [28]. Our experimental plots contained microbial communities comprised of numerous soil-dwelling taxa. Inspecting the bacterial phylum level profile in our experimental plots, we encountered some significant soil bacterial groups. *Actinobacteriota* dominated the soil bacterial community. The presence of this phylum is essential in soils, as they have vital ecological functions; they are capable of metabolising organic matter, degrading cellulose, chitin, pectin and hemicellulose; they can remove contaminants from soil such as pesticides and heavy metals [29-31]. In slightly lower abundance, *Acidobacteriota* phylum has very similar functions as organic matter degradation, as well as metabolising inorganic and organic sources of nitrogen.

Proteobacteria abundant in our plots are very common in soil environments where they are involved in many soil functions, such as C, N and S cycles [32-34]. *Chroloflexi* phylum was also prominent; these taxa are very diverse, including aerobic chemoheterotrophs, photoautotrophs and thermophilic organisms, as well as anaerobic species that reduce chlorinated organic compounds. This phylum also contains species capable of nitrification and oxidising CO_2 in significant concentrations [35-37]. This is in agreement with the findings of Jin et al. [19], who also reported that the application of humate promoted *Acidobacteriota* and *Chloroflexi,* whose species are associated with the decomposition and accumulation of complex soil organic matter. Jin et al. [19], also reported reduced microbial diversity post humate, while in our study, there were no significant diversity alterations.

Firmicutes species possess iron and sulphate reduction abilities and have a critical role in soil disease control [34,35,38]. Two *Archaea* phyla were detected. *Crerchaeota* includes a diverse group of organisms that can be extreme thermophiles, associated with the N cycle and known autotrophic CO_2 fixators [39-41]. *Thermoplasmatota* are known for their roles in C and N cycles and are critical in recycling complex organic carbon [41,42].

The most abundant genus was *Rubrobacter*, known to be radiation-resistant and heat-loving [43,44]. *Solirubrobacterales 67-14* were commonly found in disturbed and poor soils, agricultural soils, earthworms holes and associated with ant activities [45,46]. *Bacillus* is a well-known genus of plant growth-promoting bacteria [47]. Members of the genus *Gaiella* are well known as plant growth promoters as well as for having antifungal properties [48]. *Xanthobacteraceae* are known for N fixation in soil and contains many species that can reduce sulphur and hydrogen compounds [49], while *Conexibater* species are known for nitrate reduction [50].

The lack of significant increase in richness and diversity weekly or overall post Hum application indicates no new species introduced or encouraged to expand above the detection threshold with the application of K humate. This also indicates that Hum did not suppress or remove a significant number of species in the soil, this could be due to temporal fluctuations driven by the rain and drought periods. Temporal analysis of beta diversity shows rainfall's strong and variable influence on all plots, indicating subtle microbiota community changes. The rain effect on the community appears unambiguous and anticipated after drought, yet distancing of the communities after the rain event suggests a different response of microbial communities to rain in treated and control plots. The high

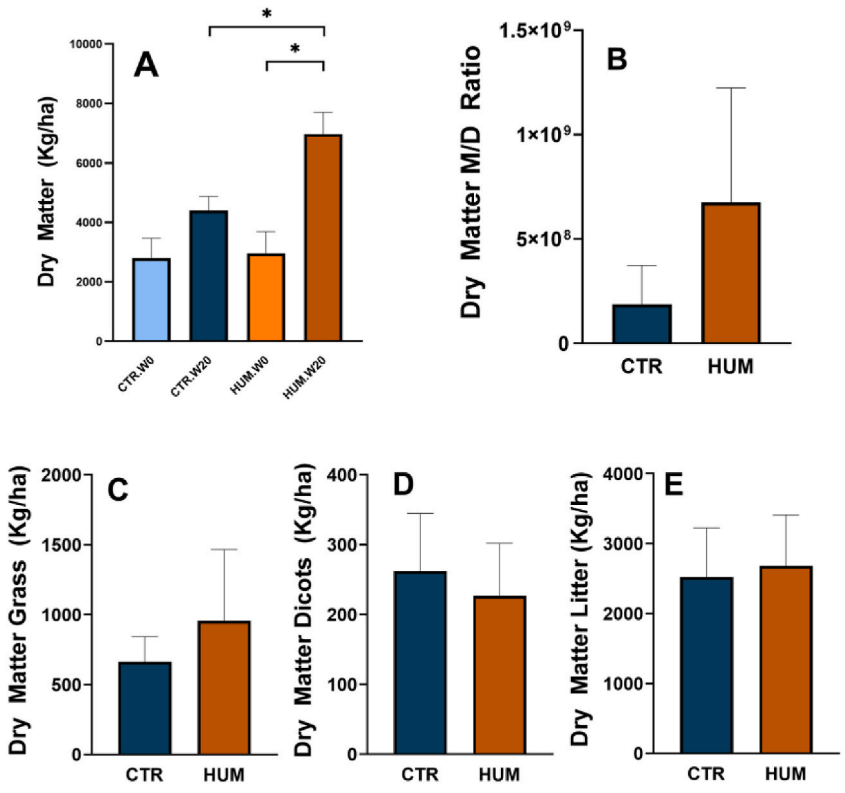

Fig. 5. Dry matter control versus Hum treated once. Panel A shows data collected at 20 weeks, and panels B, C and D data collected at eleven months post single application. Panel B shows 27.6% marginal mean increase in the grass/weed (monocots/dicots) ratio. Panels C-Grass, D- Dicotyledons and E– Litter show dry matter measured eleven months after treatment. Statistical significance is indicated with an asterisk (* = $P < 0.05$).

significance of the influence of time on microbiota may be driven by changes in the environment that fluctuated enormously (temperature, rainfall, UV radiation etc.) in Queensland over the eleven months of the experiment. Anosim multivariate analysis shows that Hum increased sample-to-sample distance, which is often a sign of a product with multiple mechanisms and variability of effects.

PD is commonly accepted as not responsive to treatments farmers tried for over a decade. Moreover, the treatments aimed to improve soil or grass health often showed initial improvement followed by a rapid decline within weeks of initial success [51]. Here we extended the experiment to eleven months post-application to observe the long-term effects on the grass. Despite the lack of statistical significance in microbiota structure alterations, there was 27.6% marginal mean increase in grass/weed (monocots/dicots) ratio and a statistically significant increase in the total number of roots in both grass and sage eleven months post-application. Although not statistically significant, M/D ratio, or increase of grass at the expense of weeds, was visually observable on the plots.

Primer Simper analysis (taxa contribution to differences between Ctr and Hum), has selected *Gaielalles* as the genus most contributing (4.22%) to the difference between Hum and Ctr, while LEfSe analysis identified this genus as a biomarker for Hum application. These bacteria are found in extreme environments and carbon metabolisers [52,53]. *Bacillus* contributing to 3.5% variation and associated with the Hum group are known plant-beneficial bacteria. On the other hand, high Simper contributing genera associated with Ctr plots included *Solirubrobacterales 6714,* found in disturbed and poor pasture soils associated with ants [54]. *Rubrobacter* (3% variation) are tolerant to high radiation and temperature [55]. They were recently reported as the most significantly increased genus in PD soils by our team [56]. Together, these four genera contribute to 15% of the differences between Hum and Ctr plots. This is very comparable to the use of sea minerals [12] and tri-sodium salt of trimercapto-S-triazine (TMT) and humate mix treatments of PD [13], which also provided several improvements in plant morphometrics and pasture yield via altering the same genera. This reproducibility could indicate that successful remediation of pasture dieback research should focus on soil microbial community restoration.

Hum treatment affected the abundance of species in the soil in a temporal fashion. Only significantly negatively correlated with time on Hum-treated plots were bacteria from the genus *KD4-96*, linked to heavy metals polluted soils [57-59]. These results indicate that Hum could be helpful in heavy metal polluted soils treatment, which was previously reported by Nong et al. [60], who demonstrated that Na humate significantly reduces toxic Cd and Pb in contaminated soils. Temporally reduced in Hum plots was also *Pseudonocardia* with antibacterial properties and linked to ant presence [61,62]. This reduction of microbiota damaging antibacterial and insect-promoting effects of *Pseudonocardia* can indirectly assist PD-affected soils.

Significantly reduced over time in control but not in Hum-treated soils was the genus *Marmoricola,* also found to be a biomarker of

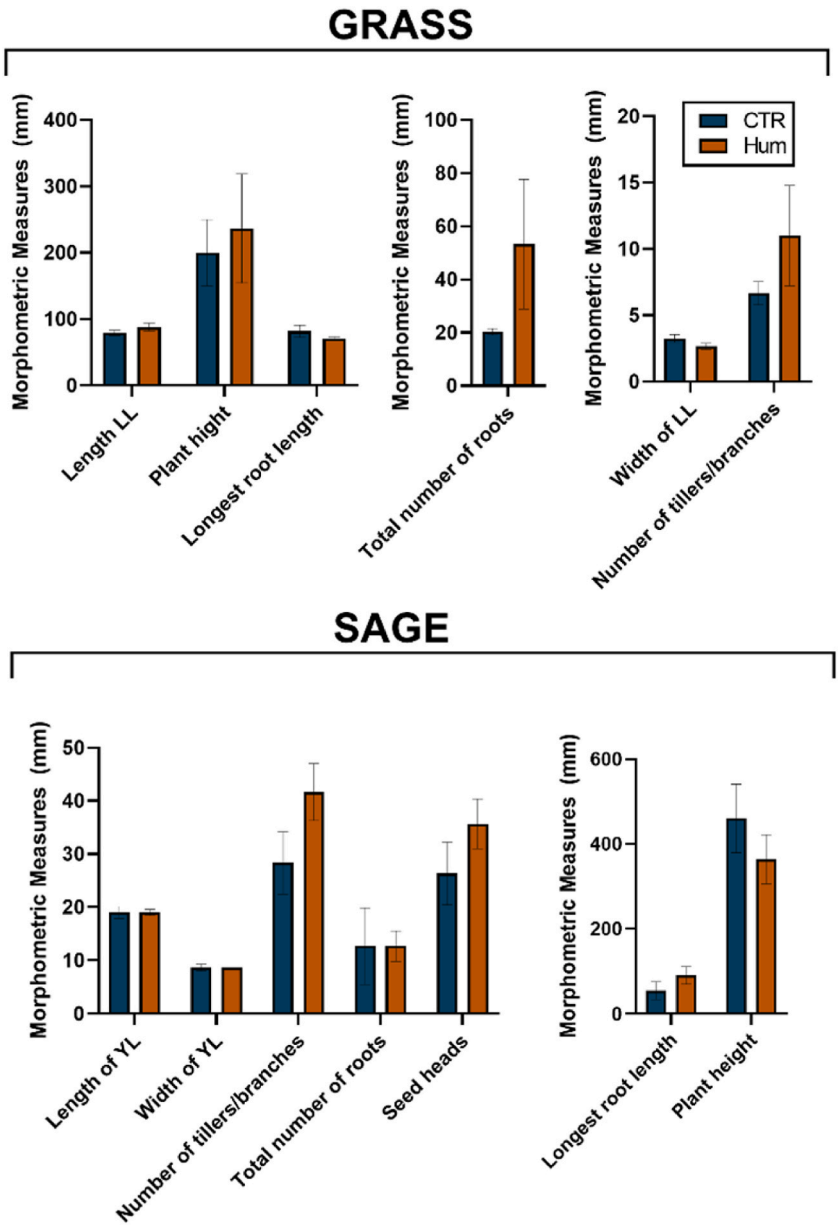

Fig. 6. Plant morphometric measurements at week 20. Control (CTR) versus potassium humate treated soil (Hum).

Pb and Zn pollution [63]. *Actinoplanes* genus is known for phosphate solubilisation [64], *Asanoa* hydrolysing cellulose bacteria [65], *BIrii 41* genus of denitrifying bacteria with a preference for arable and grassland [66,67]. *Dactylosporangium* genus has antimicrobial properties against Gram-Positive bacteria [68]. *Ellin 6067* converts ammonia to nitrite, and it is tolerant to Cd pollution [69]. The above demonstrated temporal decrease of some genera with beneficial functions for the soil and plant growth in control but not in humic-supplemented soils and may account for some of the dry mass and plant morphometric long-term benefits.

Twenty weeks after a single application of Hum there was a significant increase in dry matter compared with the control. Later post-rainfall sampling at eleven months post-application indicated an improved grass/weed ratio. Morphometrics demonstrated significant improvements in root structure in both grass and dicots eleven months post Hum application, which supports further research about the capacity of humic acids to improve pasture in long-term PD recovery. Optimising the concentration, type, and ratio of different humic acids mix could make this a viable PD treatment. Large-scale experiments are needed to facilitate the economic analysis that could justify the cost of humate with viable gains in pasture productivity.

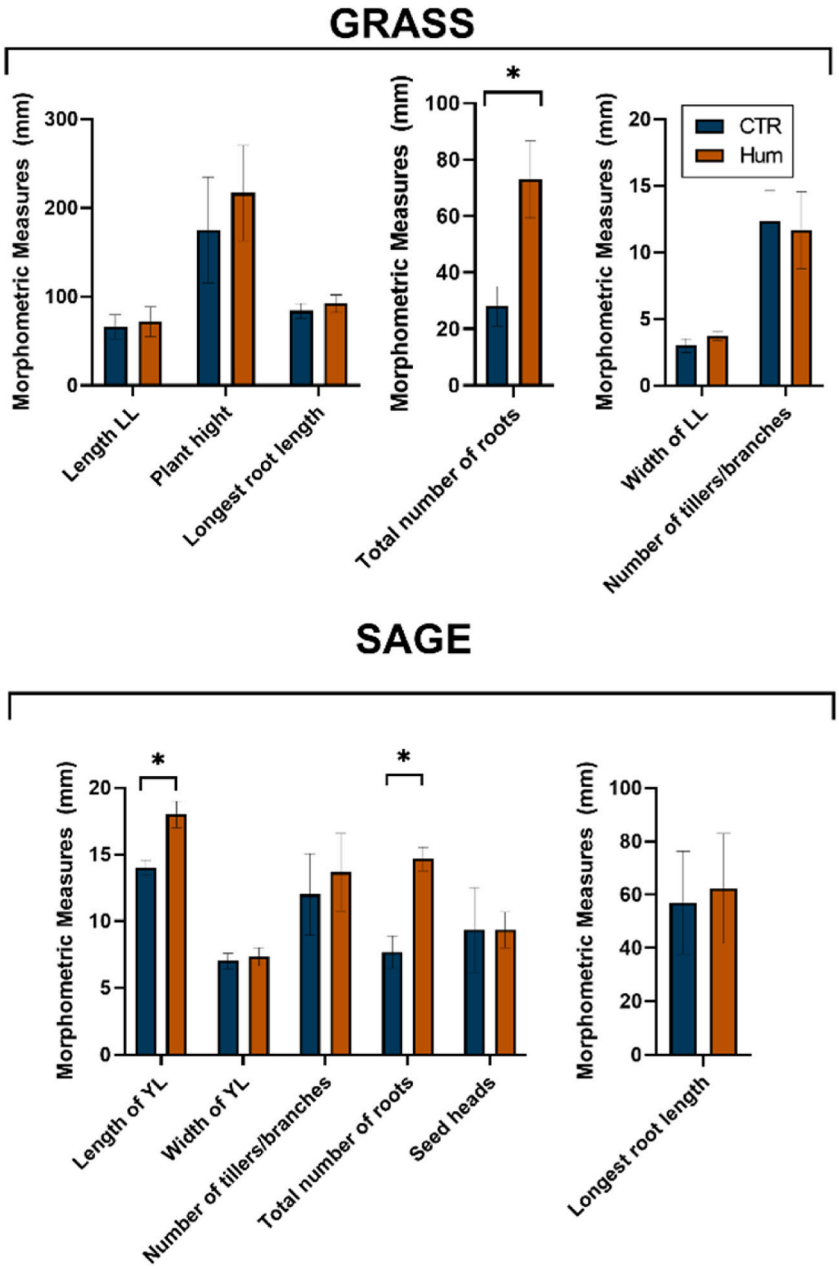

Fig. 7. Plant morphometric measurements eleven months after treatment. Control versus Hum-treated soil. Statistical significance is indicated with an asterisk (* = $P < 0.05$); (** = $P < 0.01$).

6. Conclusions

While pasture dieback is rapidly and aggressively spreading in Australia, making the pasture useless for cattle farming and affecting farmers' welfare and countries' economy, research in this area is lagging, indicating that the problem is not receiving enough attention. Here, we presented a controlled attempt at a long-term dieback recovery that showed promising results. However, until the mechanisms and environmental variables, including climate change possibly impelling the expansion of pasture dieback in Queensland and global agricultural soil desertification, are identified, we will not be able to execute more targeted and refined recovery experiments. Mechanistic studies are often high in cost and magnitude and require bringing this issue to global attention.

Declarations

Author contribution statement

Maria M. Whitton: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Wrote the paper. XiPeng Ren, Sung J. Yu and Andrew Irving: Performed the experiments; Analysed and interpreted the data, Contributed reagents, materials, analysis tools or data. Tieneke Trotter, Yadav S. Bajagai and Dragana Stanley: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data associated with this study has been deposited at Sequence Read Archive (SRA) database under the accession number PRJNA887675.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13327.

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