The effects of soil microbial community and topsoil removal on grassland restoration techniques in South Australian Mediterranean-type climate region

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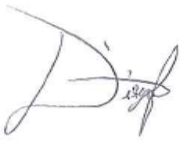
This report is provided to the Australian Flora Foundation in fulfillment of the conditions of the grant awarded to the author in August 2020. This report presents the results from a PhD study conducted by Diego R. Guevara-Torres between 2021 and 2022 on restoration techniques employing topsoil removal and microbial inoculums effects on plant and soil communities from the South Australian Mediterranean-type climate region. This study is part of a PhD supervised by Associate Professor Dr. Jose Facelli, that is aimed to be completed by July 2023 at the University of Adelaide, SA, 5005. The ideas, experimental work, results, analyses and conclusions presented in this report are entirely the author's own effort, except where otherwise acknowledged.



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Abstract

Disruptions in relationships between soil microbial communities and plants contribute to the replacement of species, affecting successional processes that can ultimately lead to the dominance of invasive species. Recent research has shown that plant-soil-microbial interactions can play a significant role determining abundance of various plants, as microbes can either favour the establishment of plants or become pathogens for them, and these effects are species specific. Hence, the management of microbial communities should be considered in restoration campaigns that aim to establish native vegetation in degraded sites. In the temperate grasslands of South Australia, the restoration of former crop paddocks (old-fields) to grasslands dominated by perennial graminoids represents a challenge due to the soil legacies caused by farming, grazing, and the dominance of invasive species. Little is known about the effect of restoration techniques on the soil microbial communities and how these could affect the establishment of native species. We conducted a glasshouse experiment using intact core soil samples from old-fields that were subject to topsoil removal and direct soil microbial inoculation, from different soil origins. We assessed the effects of the combination of these techniques on the microbial community and the biomass production of the native wallaby grass (*Rytidosperm auriculatum*). Soil physiochemical properties, plant biomass and soil microbial communities were significantly affected by topsoil removal. The use of microbial inoculum grasslands affected the number of observed microbial operational taxonomic units. The inoculum elaborated from soils dominated by native graminoids produced higher wallaby grass biomass. Our results indicate that the combination of topsoil removal and inoculums with a native origin can be beneficial to the biomass production of native grasses like wallaby grass. This study increases our understanding of the factors driving vegetation succession and represent an opportunity to improve restoration techniques.

1. Introduction

Grasslands are valuable ecosystems that have been severely impacted and modified by humans (Hautier et al. 2015). They provide forage resources for livestock grazing and essential ecosystem services such as water supply and flow regulation, carbon sequestration, soil erosion control and pollination (Bengtsson et al. 2019). Despite their importance, the impact of human activities on the environment has substantially reduced natural and semi-natural grasslands extension worldwide (Corbin and D'Antonio 2010). Moreover, the introduction of invasive plant species has degraded most grassland ecosystems by affecting their composition, structure and functionality grasslands (Vila et al. 2011). Temperate grasslands, in particular, have severely suffered from biodiversity loss and are considered as the most altered ecosystem in the world (Carbutt et al. 2017). Thus, there is a paramount need to restore grasslands and contribute to the conservation of native species.

Temperate grasslands in South Australia present a Mediterranean-type climate characterized by mild wet winters and warm to hot, dry summers (Cowling et al. 2015). Perennial and annual life cycles are two main strategies plants have developed to cope with water stress in this type of environment (Ehrlen and Lehtila 2002). Fluctuations in resources availability caused by activities such as agriculture and grazing can facilitate the establishment of exotic species (Davis et al. 2000). Exotic species with ruderal traits that enable them to overcome the local abiotic (nutrients, water availability) and biotic filters (plant and microbial community, fauna), outperforming native species (Pearson et al. 2018). As a result, exotic species can become dominant, causing profoundly changes in vegetation composition (Pearson et al. 2016).

Once invaded, it is often difficult to return native grasslands to a healthy condition. One possible mechanism underlying this may be the changes in soil conditions produced by invasive plants (Smith et al. 2021). Disruptions in relationships between soil microbial communities and plants contribute to the replacement of species, affecting successional processes that can ultimately lead to the dominance of invasive species (Reynolds et al. 2003; Inderjit and van der Putten 2010; Crawford et al. 2019). Exotic plants can alter the soil microbial community composition enhancing pathogens and producing toxins that affect native species and favours conspecifics (Crawford et al. 2019). Field and glasshouse experiments have shown promising results for soil microbial inoculation in the restoration of former arable lands (Carbajo et al. 2011; Docherty and Gutknecht 2019; Guo et al. 2019). Inoculation with soil from remnant native grasslands have shown to be beneficial for the establishment and performance of native late-successional species and detrimental for exotic species, suggesting that the origin of soil inoculum can direct the succession process to the desired community composition to be restored (Wubs et al. 2016; Smith et al. 2018a, Smith et al. 2018b). Hence, the management of microbial communities should be incorporated in restoration ecology practices (Calderon et al. 2017).

Currently, the removal of the topsoil layer is one of the most effective and used practices for restoring old-field farms in Australia and other parts of the world. However, it can be expensive and not viable in areas where geography and winds prevent the removal of topsoil due to erosion (Prober et al. 2005; Brown et al. 2017), In addition, the effects of topsoil removal are not fully understood (Brown et al. 2017). Thus, more research is needed to understand the effects of these practices on the soil microbial community.

In South Australia, the restoration of native perennial grasses represents a challenge due to the soil legacies caused by farming and grazing, the dominance of invasive species and the soil profile (Lenz and Facelli 2005; Smith et al. 2021). Perennial grasses were the dominant life form of the temperate tussock grasslands that once dominated South Australian landscape and that are currently considered as one of the most threatened ecosystems and classified as a critically endangered ecosystem (Keith 2017). Currently, invasive annual graminoids, like wild oats (*Avena barbata*), have become dominant invasive, especially in higher fertility soils (Smith et al. 2021). Experiments conducted by members of our laboratory in an old-field employing topsoil removal in a glasshouse, using soil microbial ammendments have shown promising results for the establishment of native perennials grasses (Smith et al. 2018a, Smith et al. 2018b). However, these studies did not include direct microbial manipulations. Thus, research on topsoil removal and direct soil microbial inoculation is needed to make restoration practises more effective and less expensive.

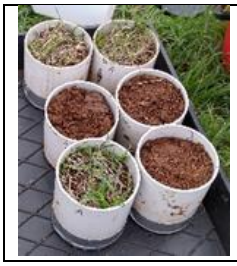
Here, we aim to determine the effectiveness of topsoil removal and soil microbial inoculation for the establishment of native perennial grasses, as well as to develop a better understanding of the relationships between native and invasive grasses and the soil microbial biome. For this purpose, we employed soil inoculum from two origins (remnant native grasslands and old-field) combined with topsoil removal.

2. Materials and methods

2.1 Experimental design

We conducted a glasshouse experiment using intact core soil samples extracted from an old-field dominated by invasive species. The sampling took place at Para Woodlands Nature Reserve (PWNR), South Australia (34.628°S, 138.785°E), a former cereal crop farmland cultivated until 2004 and currently managed for the restoration of native grasslands. PWNR is located in the Mediterranean-type climate region, characterized by dry summers and wet winters, with a mean annual air temperature of 23.6℃ and an annual average rainfall of 450 mm (Bureau of Meteorology, 2017, Fatorić et al. 2017; Hallett et al. 2018). We took the samples in June 2021, when the A. barbata and other exotic annual grasses were the dominant vegetation.

A total of 36 samples were taken in six blocks, parcels of 1 x 0.5 m, around a 20 x 20 m quadrant. Each block was subject to two topsoil treatments. The topsoil (50 mm) from half of each block parcel was removed (TSR), while the other half remained intact. Each block consisted of a battery of six intact core samples, randomly divided into two groups of three samples. One group was taken from the TSR half and the other one from the intact side with all vegetation present at the moment of extraction (Figure 1). This methodology mimics the soil condition after TSR methods are applied in restoration programs. The intact core samples were then transported to a glasshouse for further treatments and were considered as pots (10 cm diameter, 10 cm depth). Two inoculation treatments and one control were randomized within each group (see inoculation preparation below). We sowed each pot with ten wallaby grass (R. auriculatum) seeds donated by a local plant nursery (Seeding Natives). Wallaby grass is a perennial grass commonly used in restoration projects in South Australia (Jellinek et al. 2020).



Topsoil	Removed		
Inoculum	native	old-field	control
Code	TSRn1	TSRn1	TSRn1
Topsoil	Intact		
Inoculum	native	old-field	control
Code	INT1	INT2	INT3

Figure 1. Example of Block number 1

2.2 Inoculum sampling, creation, and application

We created two microbial inoculums from soil samples taken at the sampling site (old-field) and at a remnant native grassland (dominated by native species) patch located in another sector of PWNR. Henceforward these are referred to old-field inoculum and native inoculum treatments respectively. The remnant native grassland was dominated by the native perennial grass kangaroo gras (Themeda triandra) and presented a low presence of wild oats (A. barbata). Soil samples from both places were taken on the same day and transported to the laboratory for inoculum creation. Inoculums were prepared with methods adjusted from (Mardhiah et al. 2016). We prepared a microbial filtrate, containing fungi and bacteria, by diluting 110 g of soil in 1 L of reverse Osmosis (RO) water. We filtrated the suspension through a pile of sieves (2mm and 850 um) and used the slurry for microbial inoculation. Pots in microbial inoculum treatment received 100 ml of either old field or native slurries, while controls received 100 ml of RO water. Inoculum application took place three days before wallaby grass sewing and two days after (application reduced to 50 ml). Pots were located in an unheated greenhouse without artificial light at the Benham building, Adelaide, Australia (34.918°N, 138.6047°E). The experiment lasted for 110 days and harvest was done when wild oats started to seed.

2.3 Soil physiochemical sampling

Soil samples were taken by collecting representative samples of 250 g: at each block in the old-field, one sample was taken before and after the top-soil removal, resulting in six samples for each soil treatment (field samples). At the end of the experiment, one sample was taken for each pot, resulting in six samples per treatment (experiment samples). Soil physiochemical analysis was carried out at CSBP Limited (Bibra Lake, WA, www.csbp-fertilisers.com.au). The analysis included nitrate-N, ammonium-N, plant available (Colwell method) phosphorus, potassium, sulphur, organic carbon (OC), electrical conductivity (EC), and pH (CaCl2).

2.4 Plant community sampling

We registered the species present in each pot before the inoculation. Plants were harvested after 16 weeks. The aboveground biomass (biomass henceforward) of each species present in each pot was dried separately to a constant weight at 65 °C. Plant species were grouped into three main groups for further analysis based on their importance for the experiment and characteristics. The first group consisted only of wallaby grass. The second group consisted on graminoid species, mainly wo;d pats. The third group, named weeds, consisted of herbaceous exotic species (mainly *Polygonum erectum, Stellaria media and Raphanus sp*.).

2.5 Microbial community sampling

Microbial sampling in soils was conducted by collecting samples of 50 g of soil. At the sampling site, we collected one representative soil sample from the TSR and intact old-field samples, as well as one sample from the remnant native grassland (field samples). At the end of the experiment, we took one sample from each pot resulting in six samples per treatment (experiment samples). We also conducted microbial sampling from the two inoculants by collecting one sample of 50 ml for each of the inoculants. Analysis for DNA extraction, PCR amplification and sequencing for fungi and bacteria were undertaken at the Australian Genome Research Facility (AGRF, Adelaide, Australia).

2.6 Microbial data processing

Fungi and bacterial community composition were analysed separately using the phyloseq package (V1.36.0) in R (McMurdie and Holmes 2012) to calculate operational taxonomic units (OTUS) for Alpha and Beta diversity analysis. Prior to the analyses, we removed samples that had less than 1000 sequences and normalized the number of reads using the rarefy-even-depth function from the phyloseq package. The Alpha diversity was measured by the numbered of OTUS (observed) and the OTUS' Shannon, Chao1 and evenness indexes. The Beta diversity assessment was conducted on the Hellinger-transformation of the square root of the relative abundances of microbial data.

2.7 Statistical analysis

To analyse the effect of explanatory variables on dependant variables we employed linear mixed-effects models (LMMs) using the function lmer and type III ANOVA from the package lme4 (Bates et al. 2015). We assigned explanatory variables as fixed effects and block as a random effect, considering the interaction between topsoil treatment and inoculum treatment. Continuous fixed effects were scaled to facilitate model convergence. All statistical analyses were run in R software with version 4.3 R, Core Team (2020) and R Studio (version 7.2.576).

Soil physiochemical properties and plant biomass

Initially, we compared the effect of top soil treatments (TPS and intact) on the physiochemical properties from samples taken in field a beginning of the experiment using LMMs. Soil treatment and inoculum treatment were treated as fixed effects and block as a random effect. Physiochemical properties were normalized using log or square root transformations as needed. For the samples taken at the end of the experiment, we first summarized the variance of the soil physiochemical properties across treatments using a principal component analysis (PCA), using the factoextra package (Kassambara and Mundt 2017). Then we assessed the effects of physiochemical properties on plant biomass (wallaby grass, graminoids and weeds) using the Pearson's correlation coefficient to test correlations between the first two principal components and the biomass of wallaby grass, graminoids and weeds, respectively. We tested the effect of soil treatments (TSR or intact) and inoculum treatments (native, old field and control) on soil physiochemical properties in pot samples (taken at end of the experiment). We employed LMMs. Soil treatment and inoculum treatment were treated as fixed effects and block as a random effect. Physiochemical properties were normalized using log or square root transformations as needed. When significant effects were found, we performed pairwise comparisons using the emmeans function from the R package "emmeans" (Lenth 2018). We later analysed what variables could have affected plant (wallaby grass, exotic graminoids and weeds) biomass in pots samples (taken

at the end of the experiment). We employed two sets of LMMs where explanatory variables were top soil treatments (TSR or intact) and inoculum treatments (native, old-field and control) or soil physiochemical properties. We generated a ggplot2 scatter plot to visualize differences in biomass between soil treatment and inoculum treatment.

Microbial data

Fungi and bacterial community composition were analysed separately using the phyloseq package (V1.36.0) to calculate operational taxonomic units (OTUS) for Alpha and Beta diversity analysis. Prior to the analyses with pot samples, we removed samples that had less than 1000 sequences and normalized the number of reads using the rarefy-even-depth function from the phyloseq package.

Microbial Alpha diversity

The Alpha diversity was measured by the observed numbered of OTUS and the Shannon, Chao1 and evenness indexes in samples from pots, taken at the end of the experiment. We constructed LMMs and ANOVAs to test the effect of top soil treatments and inoculum treatments on most Alpha diversity measures with the exception of the observed numbered of OTUS. For the former one, we employed a generalized linear mixed models (GLMM) using the function glmer in the package lme4 (Bates et al. 2015). The same procedure was followed to the test the effect of plant biomass and soil physiochemical properties respectively. We also analysed the effects of alpha diversity measures on the biomass of plants employing GLMM. Alpha diversity measures were considered as fixed effects and plant biomass as dependent variables. Previous to modelling OTUS relative abundance was rarefied and physiochemical properties were scaled.

Microbial Beta diversity

We analysed the effect of TSR and inoculant treatments and their interactions, along with plant biomass and physiochemical properties on the variation in microbial communities' structure from pot samples (end of the experiment). For this purpose, we Hellinger-transformed the square root of the relative abundances of microbial data. The structure of microbiota communities and their relationship with the mentioned variables was analysed using the Constrained analysis of principal coordinates (CAP). Continuous factors were standardized previous to modelling process. CAP analysis was performed using the capscale function from the vegan package. An initial model considering all fixed and block as a conditioning variable was contrasted with a null model using the function ordistep from the package vegan. CAP analysis was visualized with ggplot2. The effects of fixed factors on the microbiota communities were determined by a Permutation multivariate analysis of variance analyses (PERMANOVA) based on the Bray-Curtis distances. PERMANOVA was performed using the adonis2 function in vegan applying 999 permutations and marginal effects of the terms, with block as the strata.

3. Results

3.1 Soil physiochemical properties and Plant biomass

Soils samples taken in the field at the beginning of the experiment revealed that TSR resulted in significantly higher concentrations of ammonium-N, nitrate-N, P, K, organic C and conductivity (see Annex 1 for ANOVA results). The PCA of soil physiochemical properties summarized 57.08% of the

variance in the first two principal components (Fig 3). All variables were negatively correlated to PC1 (36.8%), revealing that removing the topsoil produced a reduction in the concentration of organic C, ammonium-N, K and P. PC2 (21.01%) revealed that concentrations of P decreased in TSR samples and that nitrate nitrogen increased in some of the TSR samples. These results were corroborated by LMMs of soil physiochemical properties which found top-soil treatment as a significant variable for ammonium-N, P, K and organic C. Inoculum treatment did not present any clear of effect, except for conductivity.

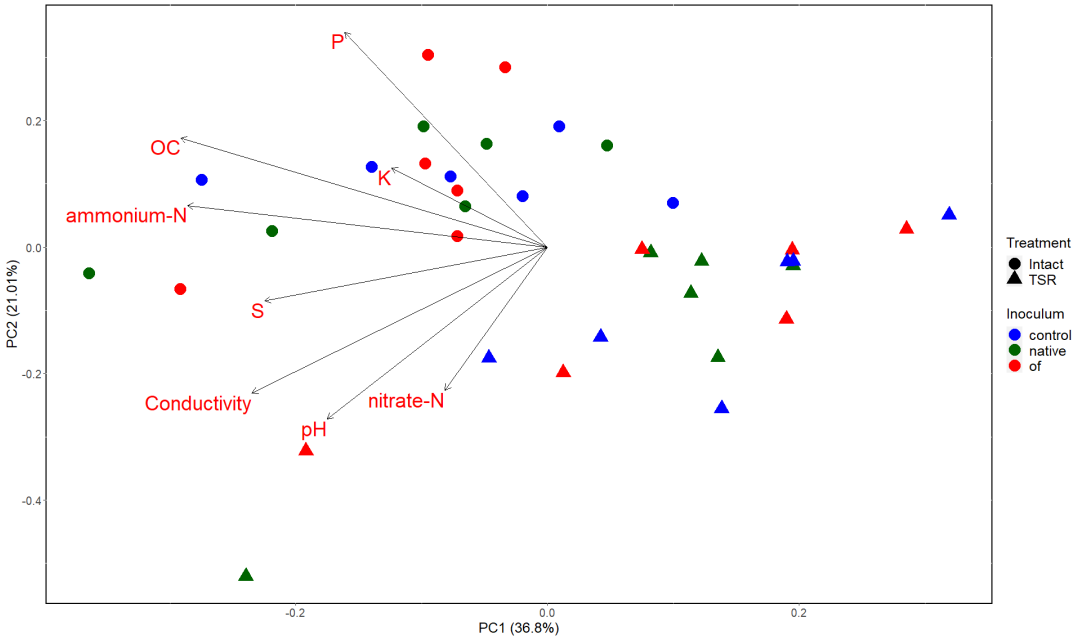


Fig. 3 Principal coordinates analysis of soil physiochemical properties in pot samples at the end of the experiment (N = 112). Total variation explained by principal component (PC) one and two is 57.8%. Top soil treatments: TSR and intact. Inoculum treatments: native in green, red old-field in red and control in blue.

Plant biomass presented weak correlations ($R < 0.7$) with the principal components and only presented a significant correlation between wallaby grass and PC2 ($r = 0.34$, $p < 0.05$), showing that wallaby grass grew better in TSR samples with lower P and more nitrogen ammonium (Fig 4). These results were partially supported by LMMs of biomass. S and organic C had a significant effect on wallaby grass biomass, but only organic C was significant in the ANOVA (ANOVA, $F = 17.75$, $p < 0.001$). Ammonium-N (ANOVA, $F = 66.64$, $p < 0.001$), nitrate-N (ANOVA, $F = 9.08$, $p < 0.01$) and P (ANOVA, $F = 16.82$, $p < 0.001$) had a significant effect on graminoids biomass. Only K had a significant effect on weed biomass but was not significant in the ANOVA. Block did not present significant effects at ANOVAS.

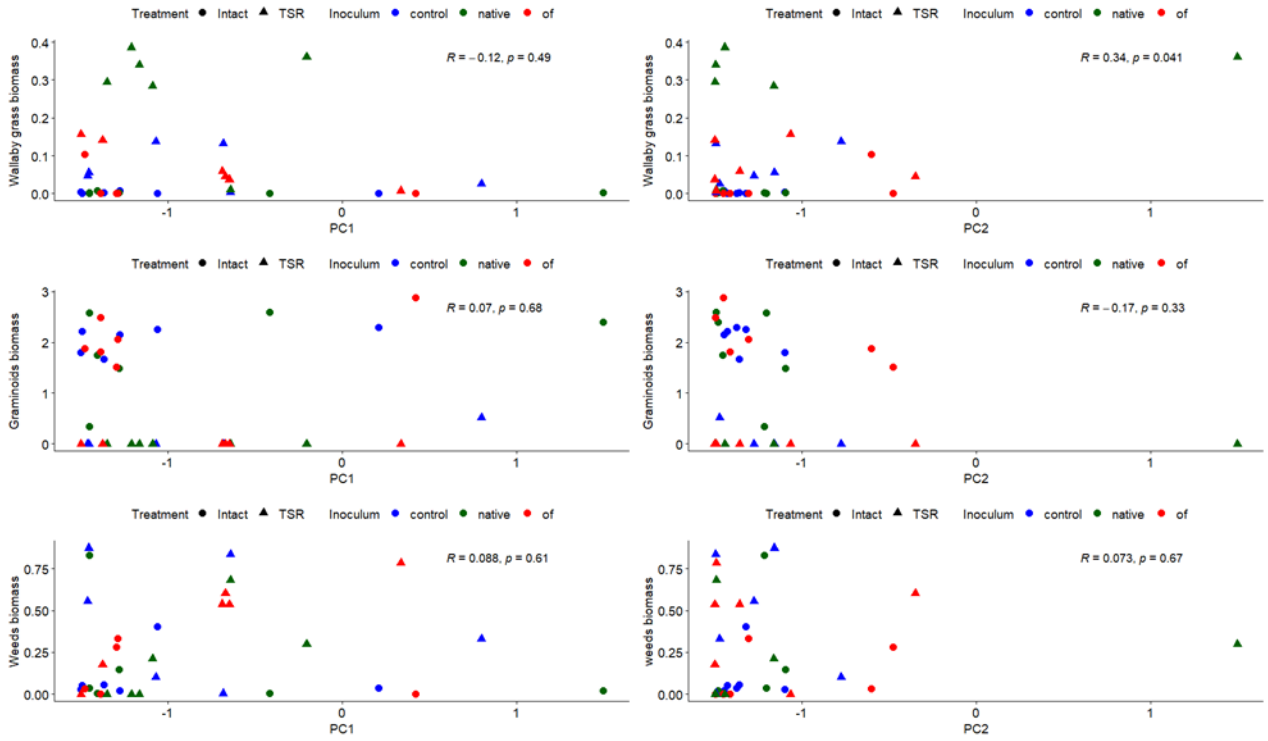


Fig. 4. Correlations between plant biomass (wallaby grass, graminoids and weeds biomass) taken in 36 pots at the end of the experiment and the first two principal components from the PCA analysis of soil physiochemical properties. Top soil treatments marked in triangles for TSR and circles for intact. Inoculum treatments coloured in green for native, red for old-field (of) and blue for control.

Plant types presented different patterns in their biomass production. The highest biomass of R. auriculatum was found in TSR pots with native inoculum application. Graminoids were mainly absent in TSR pots and weeds presented higher biomass in the TSR pots (Fig 5). LMMs of biomass showed that top-soil treatment had a significant effect on the biomass of wallaby grass (ANOVA, $F = 40.68$, $p < 0.001$), graminoids (ANOVA, $F = 221.452$, $p < 0.001$) and weeds (ANOVA, $F = 8.07$, $p < 0.01$). In the case of wallaby grass the combination of TSR and native inoculum also had a significant effect (ANOVA, $F = 12.06$, $p < 0.01$). Block did not present significant effects at ANOVAS.

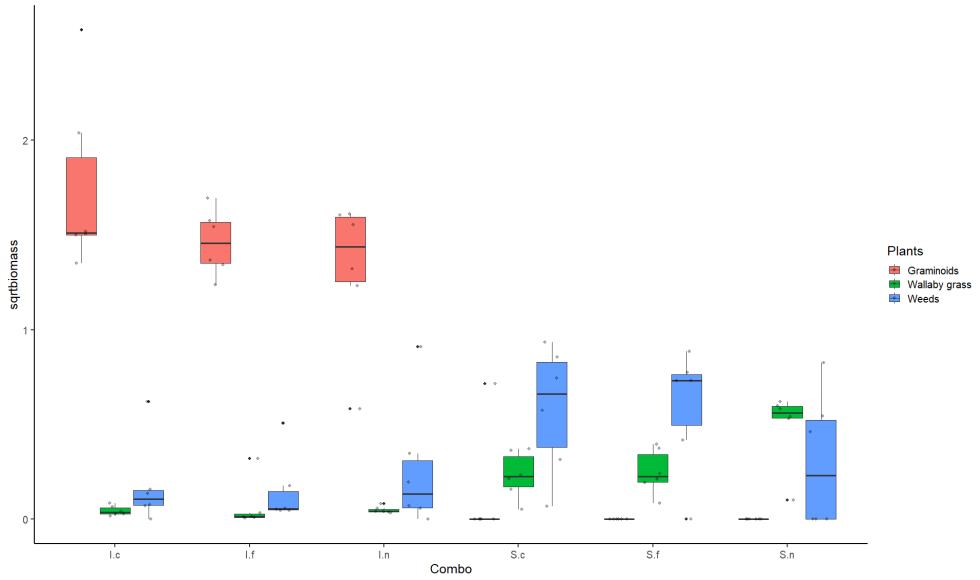


Fig. 5. Square root of biomass of wallaby grass in green, graminoids in red and weeds in blue, taken in 36 pots at the end of the experiment.

3.2 Microbial community

The sequencing efforts for fungi community resulted in 5905 OTUS found in 56 samples. The taxonomic assignment identified 14 phyla, 44 classes, 93 orders, 203 families, and 399 genera. Fungi community in pots resulted in 36 filtered, high quality samples with 13 phyla, 37 classes, 83 orders, 173 families, and 293 genera. In the case of bacteria community, the sequencing resulted in 14222 OTUS found in 56 samples. The taxonomic assignment identified 34 phyla, 94 classes, 208 orders, 295 families, and 498 genera. Bacteria community in pots resulted in 35 filtered, high quality samples with 30 phyla, 86 classes, 178 orders, 259 families, and 403 genera. Sample TSRo5 (topsoil removed with old-field inoculant from block number 5) from the bacteria data presented 333 sequences and was removed. All other samples presented more than 1000 sequences.

Microbial Alpha diversity

Fungi and bacteria alpha diversity measures were calculated on pot samples at the end of the experiment to assess the effect of TSR and inoculum treatments (Fig 6). Block represented a small portion of the variability in the linear mixed-effects models.

For fungi, generalized linear mixed-effects models showed that TSR ($p < 0.001$) significantly decreased the observed number of OTUS and that old field inoculum application ($p < 0.05$) increased it. Linear mixed-effects models showed that TSR significantly decreased Shannon ($F = 16$, num df = 1, den def = 34, $p < 0.01$), Chao1 ($F = 48.38$, num df = 1, den def = 29, $p < 0.001$), and evenness ($F = 9.77$, num df = 1, den def = 34, $p < 0.001$). In the case of bacteria, TSR significantly decreased the observed number of OTUS ($p < 0.001$), Shannon ($F = 13.26$, num df = 1, den def = 29, $p < 0.001$), Chao1 ($F = 6.92$, num df = 1, den def = 28.25, $p < 0.05$), and evenness ($F = 14.08$, num df = 1, den def = 33, $p < 0.001$). The interaction of TSR and inoculum also had a significant effect for the observed number of OTUS ($p < 0.001$) and Shannon diversity ($F = 5.04$, num df = 1, den def = 29, $p < 0.05$).

The analysis on plant types biomass effects on fungi alpha diversity measures showed that graminoids significantly increased the observed number of OTUS ($p < 0.001$), Shannon diversity ($F = 14.45$, num df = 1, den def = 34, $p < 0.001$), Chao1 ($F = 33.44$, num df = 1, den def = 30.56, $p < 0.001$), and evenness ($F = 7.96$, num df = 1, den def = 34, $p < 0.01$). Likewise, graminoids had a positive effect on bacteria observed number of OTUS ($p < 0.001$), Shannon diversity ($F = 5.69$, num df = 1, den def = 33, $p < 0.05$), Chao1 ($F = 4.63$, num df = 1, den def = 29.5, $p < 0.05$), and evenness ($F = 8.8$, num df = 1, den def = 33, $p < 0.01$). Wallaby grass ($p < 0.05$) and weeds ($p < 0.05$) also had a positive effect on the bacteria observed number of OTUS.

Alpha diversity analysis for soil physiochemical properties (P and ammonium-N due to correlation) showed that K ($p < 0.001$) and organic C ($p < 0.001$) had a positive effect while S ($p < 0.05$) and conductivity ($p < 0.001$) significant negative effects on fungi observed number of OTUS. Organic C presented a significant positive effect for fungi Shannon diversity ($F = 9.36$, num df = 1, den def = 26.58, $p < 0.01$), Chao1 C ($F = 48.54$, num df = 1, den def =30, $p < 0.001$) and evenness ($F = 8.6$, num df = 1, den def = 34, $p < 0.01$). For Chao1, K ($F = 4.38$, num df = 1, den def = 31.64, $p < 0.05$) and conductivity ($F = 4.38$, num df = 1, den def = 31.64, $p < 0.05$) also presented a significant effect. In the case of bacteria, organic C ($p < 0.001$) and Ph ($p < 0.001$) presented a positive significant effect on the observed number of OTUS, but S ($p < 000105$) and conductivity ($p < 0.001$) a negative one. For Shannon diversity, S presented a negative effect ($F = 9.99$, num df = 1, den def = 32, $p < 0.01$) and organic C presented a negative effect ($F = 12.12$, num df = 1, den def = 32, $p < 0.01$). Likewise, evenness presented the same relation with S ($F = 6.56$, num df = 1, den def = 29.27, $p < 0.05$) and organic C ($F = 14.48$, num df = 1, den def = 31.13, $p < 0.001$). In the case of Chao1, organic C presented a positive effect ($F = 10.53$, num df = 1, den def = 30.25, $p < 0.01$) and conductivity a negative one ($F = 6.69$, num df = 1, den def = 29.01, $p < 0.05$).

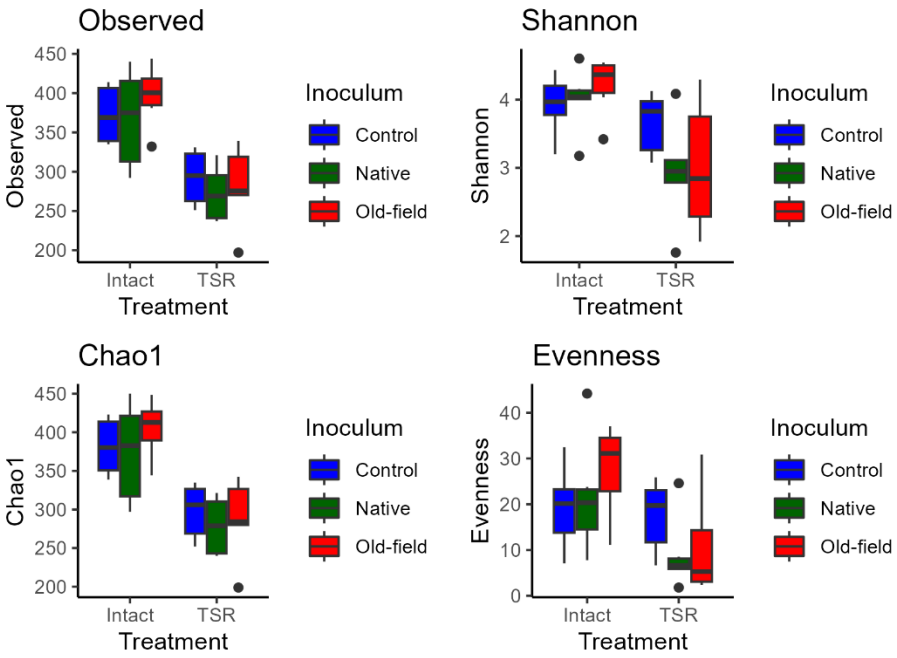


Fig 5a. Alpha diversity measures for fungi community taken in 36 pots at the end of the experiment. Top soil treatments: TSR and intact. Inoculum treatments: native in green, red old-field in red and control in blue.

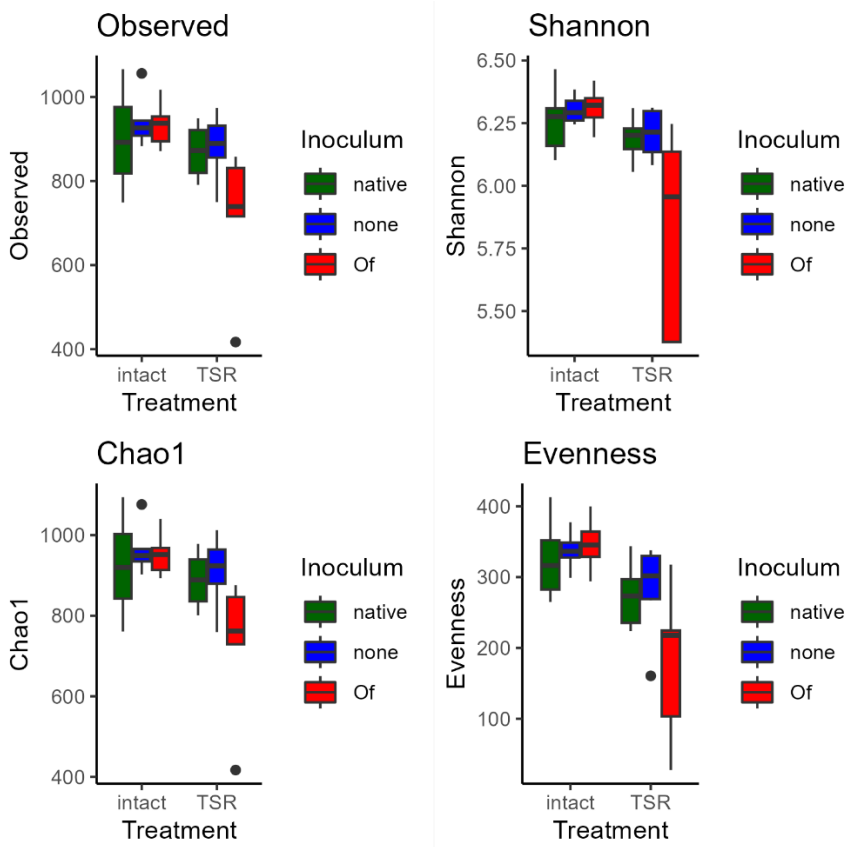


Fig 5b. Alpha diversity measures for bacteria community taken in 35 pots at the end of the experiment. Top soil treatments: TSR and intact. Inoculum treatments: native in green, red old-field in red and control in blue.

Microbial Beta diversity

CAP analysis for fungi revealed differences in fungi community structure based on significant correlations with TSR treatment, C, K and S and pH, explaining 14.7 % of the variation. In the case of bacteria, CAP analysis revealed differences based on significant correlations with conduct, TSR treatment, S and nitrate-N concentrations that explained 16.3 % of the variation. CAP biplot showed clear differences between TSR and intact samples, with wallaby grass and weeds biomass correlated to TSR samples and wild oats biomass correlated with intact samples (Fig. 6). Most physiochemical properties were correlated with intact samples. PERMANOVA results for fungi revealed that only TSR treatment (R2, 0.16, $p < 0.01$) and S (R2 = 0.08, $p < 0.05$) had a significant effect on the fungi community structure. In the case of bacteria, PERMANOVA results revealed that TSR treatment (R2, 0.2, $p < 0.01$), ammonium-N (R2 = 0.01, $p < 0.01$), and S (R2 = 0.01, $p < 0.01$) had a significant effect on the bacteria community structure.

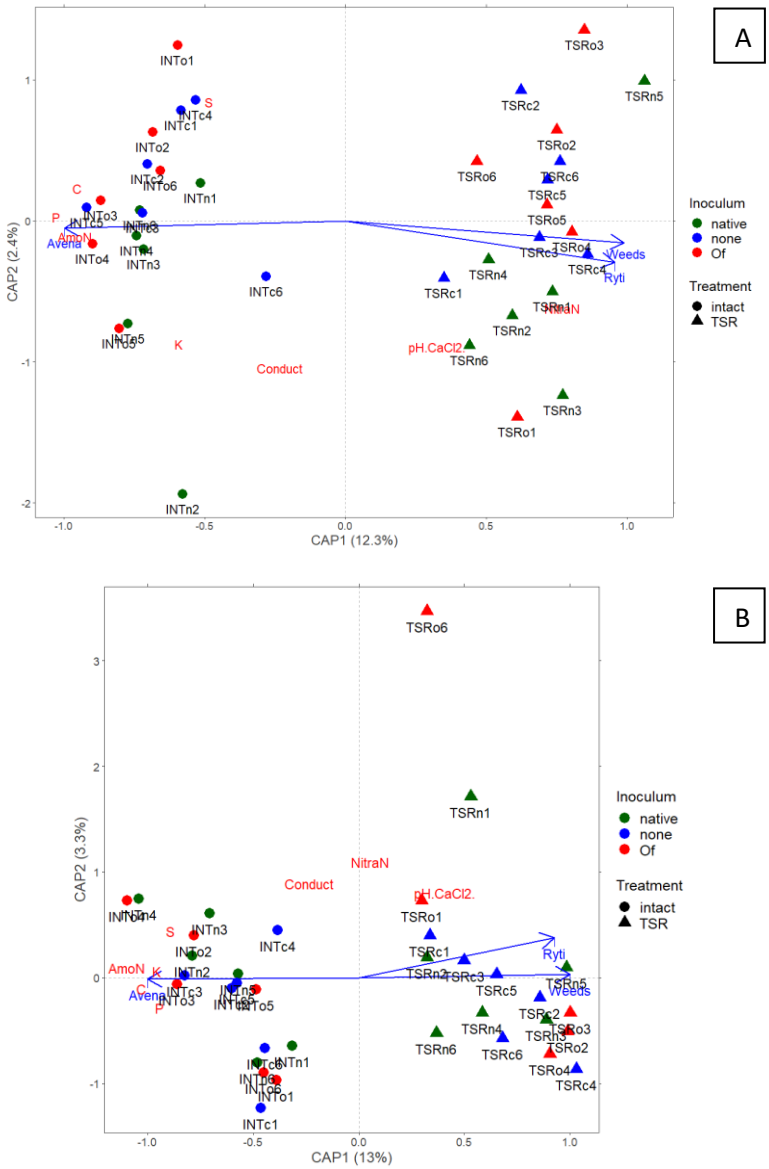


Fig 6. Constrained analysis of principal coordinates plots for fungi (a) and bacteria (b) communities. Top soil treatments marked in triangles for TSR and circles for intact. Inoculum treatments coloured in green for native, red for old-field (of) and blue for control (none). Vectors in blue represent the biomass of plant types (wallaby grass, graminoids and weeds). In red the concentrations of soil physiochemical properties.

4. Discussion

Soil physiochemical properties, plant biomass and microbial communities were profoundly affected by TSR across the experiment. In concordance with other studies, TSR effectively reduced the nutrient concentration in soil (Rasran et al. 2007; Resch et al. 2019; Zhang et al. 2021). Similarly, (Smith et al. 2021) also reported a reduction in nutrients after scalping in the same type of grasslands. Significant reductions associated to TSR were found for in ammonium-N, P, K, organic C. However, nutrients presented weak correlations with and plant biomass. Furthermore, only organic C was a significant

predictor across most alpha diversity measures of bacteria and fungi. These results indicate that TSR soils in our experiment were characterized by a decrease in nutrients, particularly in organic C.

Plant biomass clearly varied between treatments, revealing that graminoids were mainly absent once seed legacies from the topsoil were removed. A reduction in exotic annuals after TSR have been reported by other studies in Australian grasslands (Brown et al. 2017). The complete absence of dominant graminoids like wild oat (*A. barbata*) suggests that their seed bank might be present only in the topsoil layer. In contrast, the appearance of weed (exotic herbaceous species) in TSR pots, suggests that a fertile seed bank can be found in deep soil layers. Furthermore, the germination and biomass of species like Polygonum erectum, Stellaria media and Raphanus sp. might have been favoured by the absence of exotic graminoids. Likewise, wallaby grass biomass was higher in TSR pots. The dominant graminoid in our experiment, wild oat, is known to supress other herbaceous species in South Australian temperate grasslands (Lenz and Facelli 2005). Thus, it is possible that graminoids biomass played a major role in the germination and biomass production of other plant groups. Furthermore, the significant effect of graminoids biomass over bacteria and fungi alpha diversity measures suggests that the dominance of graminoids could also have affected microbial communities. Recent research has shown that the chemical composition of root exudes of wild oats can determine the microbial community assemblies by the interaction of their root exudates with the last ones (Zhalnina et al. 2018).

The microbial community also registered a significant shift in the microbial community structure of TSR pots. Similar results have been reported in agricultural and grassland systems (Wubs et al. 2016; Farrell et al. 2020; Zhang et al. 2022). Likewise, Smith et al. 2021 reported that TSR could change microbial community structure in the short term. Apart from TSR, organic C, K and S, pH and nitrate-N concentrations presented significant correlations according to the CAP analysis. However, the amount of variability explained by these variables was very low. The PERMANOVA results pointed out S and ammonium-N as significant predictors along with TSR. Nitrogen has been considered as a critical nutrient in old-fields by other studies (van der Bij et al. 2017). Contrastingly to alpha diversity GLMMs results, the biomass of graminoids was not recognised as an influential variable in the community structure analysis.

The use of inoculums only produced an effect on the biomass of wallaby grass in TSR pots, and had an effect, when combined with TSR, on the bacteria observed number of OTUs. Even though the inoculation did not generate significant effects on the rest of alpha diversity measures, we reported a certain decrease in these measures for TSR pots with the old-field inoculum. These results indicate that microbial inoculums were not effective without the application of TSR. The limited results obtained by inoculation across treatments might be associated to the short period of time the experiment lasted. Furthermore, the effectiveness of inoculums can be negatively affected by legacies from local microbial communities (resistance to new OTUS), as well as an insufficient concentration rates that secure the survival and establishment of microbes from OTUS (Perkins and Bennett 2018). The effects of the inoculation conducted in this study might not have been strong enough to produce a shift in the microbial community structure as a whole, but certainly altered the number of OTUs by increasing or decreasing the presence of certain groups. The positive effects found for the production of wallaby grass biomass demonstrate that the origin of the inoculum is crucial the inoculation effectiveness. These results are consistent with previous studies employing soils amendments taken from the same type of grasslands (Smith et al. 2018). Thus, we consider that microbes present in soils where target restoration vegetation

is dominant are required to drive microbial and vegetation communities in restoration sites to the desired direction.

5. Conclusion

In conclusion, we demonstrated that TSR can produce significantly changes in nutrient, plant biomass and microbial communities. Our results also showed that the dominance of invasive graminoids can affect plant and microbial communities. Thus, the TSR is a necessary method to efficiently removed the influence of exotic species in the system and the legacies associated to them. The opportunity created by after the removal of exotic vegetation and soil legacies by TSR, can be enhance the positive effects of the application of inoculums. Our results demonstrated that the combination of TSR and inoculums elaborated from native soils can be beneficial to the biomass production of native grasses like wallaby grass. Therefore, we recommend the application of both techniques in restoration projects.

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