Inoculum origin and soil legacy can shape plant-soil feedback outcomes for tropical grassland restoration

Running Head: Grassland restoration: inocula and legacy effects

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Abstract

Restoration techniques tailored to grasslands are needed to improve restoration effectiveness in tropical landscapes. In this work, we investigated the joint effects of plant-soil legacies and soil inocula from native and invaded Cerrado grasslands to test whether different microbial origins affect plant-soil feedbacks in ways that can foster restoration techniques. Using two grass species, we measured aboveground biomass, and several plant traits over two growing cycles. Species responded differently to inocula and legacies. The legacy of the invasive *Urochloa eminii* and invaded soil inocula positively affected mycorrhizal colonization. The legacy of *Diectomis fastigiata* resulted in a negative self-feedback discouraging its use in restoration. The invasive species success is in part due to its broad ecological niche, easily coping with soil differences. Our research points to the possibility of using soil inocula and legacies to aid restoration efforts in the tropics; allowing restorers to target desired functional trait stimulation for a given ecosystem.

Key-words: plant-soil feedback, soil inoculation, legacy effects, invasive species, restoration techniques, Cerrado.

Implications for practice

- Soil inoculation should be used for the restoration of non-forested ecosystems in the tropics as a soil improvement tool enriching the soil with local microbes
- Restoration practitioners need to consider carefully soil feedbacks, especially from annual plants, ensuring they restore the soil as well as the vegetation
- Soil legacies must be considered in restoration planning in order to generate long-term restoration success, thus maximizing cost-benefits.

Introduction

One of the main challenges of current restoration efforts in tropical areas is to develop cost-effective techniques for the restoration of non-forested landscapes (Silveira et al., 2020). Most of the restoration knowledge currently available was designed for forests (Overbeck et al, 2013); and temperate ecosystems (Hartemink 2002) knowledge for grasslands and particularly tropical grasslands is largely lacking (Overbeck et al. 2015). At the present, many techniques are being tested in tropical grasslands, from direct seeding (Coutinho et al. 2018), to top-soil transfer (Pilon et al. 2019), with varying degrees of success and applicability.

A relatively novel restoration technique being explored is the use of microbes to foster restoration initiatives (Kardol et al. 2009; Wubs et al. 2016), given they affect plant-performance above and belowground (Kardol & Wardle 2010). Top-soil inoculation is an example of such technique to improve restoration (Wubs et al. 2016); consisting on the transfer of small amounts of top soil, containing microbiota from a natural area, to an area that will be restored. It considers that the transfer of such microbes is capable of stimulating native plant regeneration. Its theoretical framework postulates that microbes present in the soil are capable of improving plant colonization through multiple pathways, from pathogen suppression to growth stimulation (Ortíz-Castro et al. 2009). Soil inoculation has been used as an effective tool to promote growth of target grassland species in the Netherlands (Carbajo et al. 2011), where it has been shown that inoculant origin is capable of directing final desired environments towards grasslands or heathlands, demonstrating the strong role microbes can have in community assembly (Wubs et al. 2016).

Associations developed by plants with microbes during their lifetime which can last in the soil even after the focal plant has died, termed legacy effects (Grman & Suding 2010), mean the colonization history of a soil patch can influence the future outcomes of plant succession (Kardol et al. 2007). Legacy effects act on soil physical, chemical,and biological properties (Kardol et al. 2007; Corbin & D'Antonio 2012). Soil legacies can be an important factor regulating plant community dynamics, hence, they can be used to target plant community development improving the management of restored ecosystems (Wubs et al. 2019). Wubs et al. (2019) have shown that in temperate systems, plant community composition has long-lasting effects on both plant and soil communities.

Plants grow in the presence of several groups of organisms in the soil, from bacteria to fungi (Compant et al. 2019); altering microbial populations due to associations they develop during their lifetime (Haichar et al. 2008); and developing positive, negative or neutral feedbacks (Hassani et al. 2018). Of particular interest in the tropics are the arbuscular mycorrhizal fungi (AMF), which allow plants to obtain nutrients from the soil, besides helping in soil water flow (Delavaux 2017). Moreover, soil microbial populations are capable of stimulating biomass production (Bailly & Weisskopf 2012) and pathogen suppression (Beneduzi et al. 2012) or build-up (Packer & Clay 2002). Therefore, microbes can be great allies in restoration ecology (Trevelline et al. 2019), for instance, AMF inoculation in restored plots yields a gain in biomass averaging 1.7 times that of non-inoculated plots (Neuenkamp et al. 2018).

Soil inocula from beneath invaded and native plant communities have both been demonstrated to be biomass enhancers compared to sterilized soils (Phillips et al. 2020). Invasive species usually tend to develop more positive feedbacks compared to native species (Klironomos 2002), perhaps explaining in-part their success in biological invasions. Many factors influence the interplay between plants and microbes. For instance, late successional species are more likely to be positively affected by feedbacks with soil microbes, whereas early successional plants and annuals tend to be negatively affected (Middleton & Bever 2012). This happens most likely due to a trade-off in resource investment between defenses and growth (Lind et al. 2013), making these early-successional species more susceptible to pathogens, (Kardol et al. 2006) which accumulate over-time.

It is possible to develop strategies that encompass plant-microbial interactions to reach the desired final ecosystems of interest, specially the non-forested ones. These techniques could improve current common Cerrado grassland restoration techniques, like direct seeding (Coutinho et al. 2018). Within existing direct seeding initiatives in the Cerrado *Diectomis fastigiata* (Sw.) P.Beauv, a cryptogenic pantropical annual species (PWO, 2020; CAD Welker, Universidade Federal de Uberlândia, Uberlândia, MG, personal communication), is commonly used and dominates the plant cover in the beginning of grassland restoration (Coutinho et al. 2019). Restoration plots are also jeopardized by the invasion of African grasses, noteworthy *Urochloa eminii* (Mez) Davidse (Pivello et al. 1999; Coutinho et al. 2019). Currently no studies have investigated the role which soil inoculation

and legacy effects could have on promoting the successful growth of native over non-native grass species.

Given the importance of understanding plant-microbial interactions, we investigated the effects of native and invasive soil inocula and of sterilized soil, upon *U. eminii* and *D. fastigiata* analyzing functional traits and using aboveground biomass as a proxy for performance. We also investigated both species legacy effects in order to understand the different outcomes the species can develop in terms of soil conditioning, and if their presence can be detrimental or beneficial for restoration efforts. We hypothesized that the native inoculum would be beneficial for the species used in restoration, and that the invasive species would be indifferent to inoculum origin, both species should be negatively affected by plain sterilized soil due to a lack of microbial diversity in them. The annual *D. fastigiata* should be negatively affected by its own-legacy due to a build-up of pathogens and annual species generally presenting trade-offs between defenses and growth. The invasive *U. eminii* species should be favored by its own-legacy, as invasive species usually present self-positive-feedback. Regarding inoculum-legacy interactions, we believe the interactions will lead to multiple different effects depending on treatment combination, with an overall less pronounced effect on the invasive *U. eminii*.

Methods

Soil sampling

Soil was collected at the Estação Ecológica de Itirapina (EEI), a protected area where scientific experimentation is encouraged. The area is a sandy-soil Cerrado remnant located in the middle of the state of São Paulo, Brazil, in November 2018. In total 570 kg of soil was collected from the upper 20 cm of a firebreak, which are areas around the park devoid of vegetation, which inhibit the spread of fire. No vegetation grows on firebreaks due to constant soil upturning and management. Native soil was collected at the EEI, from a native grassland, containing several grass species, although no *D. fastigiata* were readily observed at the collection site. The invaded soil was collected from a highly disturbed patch at EEI, heavily colonized by *U. eminii*.

The soil was put in five liter pots, lined with bidim® blanket to avoid soil leaching, and finally transferred to six liter plastic bags. Pots were then transported to the

Instituto de Pesquisas Energéticas e Nucleares (IPEN), at the Universidade de São Paulo (USP), where they were sterilized with gamma radiation, in a Cobalt-60 Irradiator, with effective minimal radiation of 40 kGy according to Buchan et al. (2012). One day prior to the beginning of the experiment, soil beneath native grassland and beneath a grassland invaded by *Urochloa eminii* was collected in order to inoculate pots.

First phase of the experiment: inoculation and soil conditioning

A total of 96 pots were prepared with 6 kg of gamma-irradiated soil each, to each 32 pots we inoculated 120 g of native soil, 120 g of invaded soil and finally 120 g of sterilized soil, accounting for soil manipulation. After inoculation half the numbers of pots with each inoculum (16) were sown with *Diectomis fastigiata* seeds, a species used in direct seeding Cerrado restoration efforts (Coutinho et al. 2018). The other half were sown with *Urochloa eminii* seeds, an African invasive species widespread in Brazil (Pivello et al. 1999). Pots were watered daily and thinned out to six individuals. Pot position was randomized in the greenhouse every three weeks. Plants were grown at the greenhouse under natural light and mean daily temperature conditions - for Campinas this is 22.1º C in January and 15.7 in July, the shortest days of light in winter have less than 12 hours of light and the longest light days in the summer have more than 13 hours of light. After 14 weeks, plants were collected and the two tallest individuals from each pot were selected for subsequent functional attributes and biomass analyses.

Second phase of the experiment: soil legacies

After removing all individuals from each pot, each treatment was divided in two and each half was sown with either *U. eminii* or *D. fastigiata* in 8 pots for each treatment in May 2019. In the second week after sowing, when seedlings emerged, a small wire was placed next to the tallest individual in the pot, to mark the individual for continuous growth follow-up, thinning of plants occurred as previously cited, collecting the marked individual and the tallest individual left in the pot. The experimental design is shown in figure 1.

Aboveground biomass and functional traits measurements

Two individuals from each pot were used for analyses, thus, each pot had two repeated measurements. Functional traits were measured according to the standard methods described in Pérez-Harguindeguy et al. (2013).

Aboveground biomass

Aboveground biomass was measured collecting the aboveground part of the selected individuals (leaves and rhizome) after harvesting the plants. Leaves were scanned to measure leaf mass per area and then dried. Rhizomes and excess leaves were stored in a paper bag and left to oven dry at 60 ºC for three days, when they were weighed. Leaves and rhizome weight were then added to compound total aboveground biomass.

Leaf mass per area (LMA)

In the first phase, all leaves of the two tallest individuals in each pot were collected, and in the second phase of the experiments, LMA was obtained using four leaves from each of the two individuals measured in each pot.

Specific root length (SRL)

Root sampling happened through gently excavating the soil beneath the same two selected individuals following the roots in order to avoid breaking them, and keeping a high degree of certainty regarding root origin. For scanning, roots were placed in a transparent plastic tray covered with a water film and then scanned and analyzed with WinRHIZO^{TM} Pro 2017a software.

Plant size

Plant size was measured as number of leaves, final plant height and number of clones. All leaves were counted in each one of the two selected individuals; plant height, which was measured just before plant harvesting, was done from the base of the plant in the soil to the highest point of the plant; and number of clones, which were counted as plants were thinned.

Arbuscular Mycorrhizal Fungi colonization (AMF%)

Root sampling for mycorrhizal colonization analysis was conducted after sampling for SRL. Fine roots were collected in each pot, regardless of which individual they belonged to, to a total of at least 30 cm of roots per pot whenever possible. Six samples from each treatment in the first phase and four samples from each treatment in the second phase were collected. Methodology for staining is available in the supplementary material.

Soil analyses

Soil samples were sent to the Department of Soil of the Universidade Federal de Viçosa (UFV) for analysis. Air-dried soil samples from three pots per treatment in the first phase of the experiment and mixed soil samples from three pots for the second phase of the experiment were collected and sent for analysis at the soil laboratory at UFV. Methodologies used for soil measurements are available in the supplementary material

Statistical analyses

In order to understand general plant responses to the treatments, we performed Principal component analyses (PCA) jointly and separately for each. In order to compare variables among treatments, we performed bootstrap analyses. All variables were compared only between pairs of the same species, within the same experimental phase (conditioning or legacy phase). The metric used consisted in the mean group difference, repeated 10.000 times, creating a normal distribution, which can be compared to the measured values.

Data were analyzed using R software version 3.6.1. (R Core Team 2013). Packages ggplot2 (Wickham 2016) and FactoMineR (Le et al. 2008) were used for data visualization. Packages Rsampling (Prado et al. 2016), shiny (Chang et al. 2020) and PerformanceAnalytics (Peterson & Carl 2020) were used for performing bootstrap analysis in order to make pair-pair comparisons.

Results

Conditioning phase

A PCA with all the treatments and all traits from the conditioning phase (Figure 2) generated a first axis of (PC1) explaining 56.2% of the variation and PC2 accounting for 25.1%, together they represented 81.3% of the variation. *Diectomis fastigiata* and *U. eminii* were separated along the second axis of the PCA. *Urochloa* was mostly dispersed in the positive half whereas *Diectomis* dispersed mostly in the negative half. *Diectomis* tended towards higher height and biomass values, and *U. eminii* tended towards higher LMA, especially in the sterilized-soil treatment. *Diectomis fastigiata* in sterile soil showed the highest SRL values, segregating from other *D. fastigiata* treatments, towards negative PC1

values, meaning a lesser investment in biomass production. The PCA shows the two species allocate resources in different ways; hence, each species was hereafter treated independently.

We observed that *D. fastigiata* performed better when inoculated with living soil than when grown on sterilized soil, presenting higher biomass and height values ($p<0.0001$) (Figure 3A; Table S1). *Diectomis fastigiata* grown in the sterilized treatment showed smaller values for all the measured traits, except values, when compared to both invaded and native soil inoculum treatments (Figure 3-E). Mycorrhizal colonization could not be compared to the sterile treatment due to insufficient root production.

Regarding the growth and functional traits of *U. eminii*, LMA values were different among all three treatments (Table S1), being highest for the sterile treatment ($p<0.0001$), followed by plants grown with the invaded ($p=0.011$)and then the native soil inoculum ($p=0.017$). *Urochloa eminii* grown with native soil inoculum showed higher SRL values compared to the other treatments ($p=0.04$). Although grown in completely sterilized soil, *U. eminii* had much lower mycorrhizal colonization than the other two treatments ($p < 0.0001$). No other differences were observed amongst *Urochloa eminii* treatments

Regarding soils, after the first phase, they tended to be grouped by inocula rather than species, the exception being the invaded soil inoculum treatment which dispersed along the second axis of the plot, with a high variation in cation exchange capacity (T) (Figure 4). PC1 accounted for 64.1% of the variation whereas PC2 accounted for 19.4%, 83.5% of the variation in soil could be explained by these two axes. Sterilized soils had the highest levels of phosphorus and tended at the same time to have lower values of sum of bases (SB), base saturation (v) and pH measured with water ($pH.H_2O$), opposing native soil inoculum treatments, values are shown in table S4.

Conditioned phase – Legacies

Survival of individuals in the legacy phase is given in table 1. A PCA regarding soil chemistry explained 71.2% of the variation with axes 1 and 2 only. *Diectomis fastigiata* and *U. eminii* soils segregated mostly along the first axis of the PCA, *D. fastigiata* towards the negative side of PC1 and *U. eminii* towards the positive side (Figure 5). Soils where *U. eminii* grew in the second phase were concentrated towards positive PC1 and PC2 axes, the exception being *U. eminii* soil with *D. fastigiata* legacy, which sided with soils where *D.*

fastigiata had grown, towards negative PC1 values. Soil P and pH.H_2O were the variables that segregated soils the most, phosphorus was negatively associated with pots where *U. eminii* was grown in the second phase. All the three treatments located within extremes of the figure had *D. fastigiata* grown in the second phase, the treatments were *Diectomis* legacy with sterilized and invaded soil inocula, and *Urochloa* legacy with invaded soil inoculum. We can see that *U. eminii* legacies scatter around lower base saturation (v) and sum of bases values. Values are shown in Table S5.

Diectomis fastigiata

Diectomis fastigiata with self-legacy and native soil inoculum had lower trait values compared to all other treatments for: aboveground biomass, height, number of leaves and AMF% (Table S2). *Diectomis fastigiata* grown in un-inoculated sterilized soil with *U. eminii* legacy showed the highest values of aboveground biomass, LMA and number of leaves compared to all other treatments (Figures 7; S1). Height of *D. fastigiata* was also higher in sterilized soil inoculum with *U. eminii* legacy than in all treatments except for *D. fastigiata* legacy with invaded soil inoculum (Figure S1). The sterile inoculum with *U. eminii* legacy did not differ in mycorrhizal colonization compared to the other *U. eminii*-legacy treatments (Table S2).

Native soil inoculum was associated with lower mycorrhizal colonization (Table S2) of *D. fastigiata* roots ($p<0.0001$). *Diectomis fastigiata* legacies seem to be associated with higher SRL values when compared to *Urochloa* legacies with living soil inocula (Table S2; Figure 6). Growing with native soil inoculum and self-legacy, *D. fastigiata* presented the smallest values of AMF%, whereas with self-legacy and invaded soil inoculum, AMF% was higher than for *Urochloa* legacy with native inoculum and equal to the other *Urochloa*-legacies treatments (Figure 6).

Urochloa eminii

In *Urochloa* most differences were seen in comparison to the sterilized soil inoculum treatments. Self-legacy with sterilized soil rendered higher aboveground biomass, LMA and height values compared to all pots with *D. fastigiata* legacy, and at the same time, also presented lower SRL values (Table S3). *Urochola eminii* grown with *D. fastigiata* legacy in sterilized soil presented lower aboveground biomass than all other *U. eminii* legacies

treatments, as well as lower LMA and height than *U. eminii*-legacies with invaded and sterilized soil inocula (Figures 7, S1). The *U. eminii* treatment with *D. fastigiata* legacy and sterilized soil inoculum also showed similar AMF% compared to *U. eminii* legacy in sterilized soil and *D. fastigiata* legacy with native soil inoculum.

Urochola eminii with *Diectomis* legacy and invasive soil inoculum had higher SRL than all *Urochloa*-legacy treatments (Figure 7), and the only difference when compared to other *D. fastigiata* legacies was in mycorrhizal colonization, which was higher ($p<0.0001$) (Table S3). *Diectomis fastigiata* with both native and sterilized inocula showed lower AMF% levels than all other *Urochloa* legacy treatments, including sterile *U. eminii* and *D. fastigiata* legacy with invaded soil inoculum. The latter and the *U. eminii* legacies with both native and invaded soil inocula (i.e. living soil inocula), responded in a similar way in respect to AMF%, the three being equal among themselves, presenting higher values of AMF% than the other treatments (Figure 6).

Discussion

Soil legacies and inoculum origin are capable of altering plants responses to the environment. *Diectomis fastigiata* was benefitted by soil inoculation. Each species legacy was different, and stimulated different traits. *D. fastigiata* legacies stimulated finer root production while *U. eminii* legacies stimulated mycorrhizal colonization, pointing to the possibility of using plant legacies as drivers for desired functional trait goals in restored ecosystems. *Urochloa eminii* and *D. fastigiata* grown in sterilized soil with *U. eminii* legacy presented the highest average aboveground biomass values, indicating some grasses might be benefitted by lower diversity inocula. The origin of the inoculum affected the net feedback response; *D. fastigiata* was negatively affected by its own legacy; an effect that was overcome by the presence of invaded soil inoculum, with roughly the same aboveground biomass production compared to those grown with *U. eminii* legacies. Overall results indicate that the presence of *U. eminii* with its subsequent removal should not constitute an impediment for the establishment of fast-growing grasses in ecological restoration regarding its microbial legacy effects. Legacies and inocula are possible tools to be used in tropical settings aiding grassland restoration techniques. However, further studies are needed to evaluate the legacy effects of exotic grasses on the establishment and growth of native slow-growing grasses.

Soil inoculation effects and mycorrhizal responses

In the conditioning phase, we aimed to test if the origin and presence of the inocula affected plants, more specifically, if native soil inoculum would benefit *D. fastigiata*, a species used in ecological restoration projects in Neotropical savannas (Coutinho et al. 2018; Sampaio et al. 2019). *Diectomis fastigiata* was benefitted by the presence of soil inocula regardless of their origin, with soil sterilization hindering its development. This is in line with recent evidence provided by Phillips et al. (2020), where a native Chaparral shrub was indifferent to inoculum origin, although they do not propose mechanisms for this. We believe that the presence of microorganisms positively affects *D. fastigiata*, allowing it to obtain nutrients more effectively, the presence of one mycorrhizal species alone (*Glomus intraradices*) is capable of transferring organic N and stimulate plant growth (White et al. 2015). Soil inocula here likely had thousands of different microbial and fungal strains, which likely helped plant nutrient acquisition and development.

Negative effects from soil sterilization were amplified in the legacy phase, leading to a strong negative feedback, which induced high plant mortality. *Diectomis fastigiata* probably managed to grow more in the conditioning phase because of the nutrients released from the microbial biomass during the sterilization process (McLaren 1969), or nutrients available in the soil. Lacking microbes to cycle and make nutrients available, nutrient concentration availability of the sterilized treatment diminished with time due to plant uptake, leading to nutrient depletion (Bennett & Klironomos 2019) and poor plant development. It is likely that, as other plants, *D. fastigiata* needs to associate with microbes in order to obtain a satisfactory mineral nutrition from recalcitrant sources (Jacoby et al. 2017).

Urochloa eminii was largely indifferent to inoculum origin, as predicted, yet surprisingly, sterilized-soil self-legacy benefitted *U. eminii* plants the most, even with lower AMF% compared to living inocula treatments. Mycorrhizal colonization in the sterilized treatment by itself indicates that contamination occurred, as it was expected to have no colonization. Contamination by mycorrhizal fungi likely originated in fine dust present in the purchased seed mix, but most likely contamination also occurred for both species from microbes present in the seed surface as well.. Searching the literature we found three mutually synergistic possibilities to explain high biomass production for sterilized soils where *U. eminii* grew: 1) AMF spores introduced with seeds probably had a tight association developed

along the years between mycorrhizal strains and *U. eminii* in the seed production site, as specialization occurs as time goes by, with selection of preferred strains, leading to more efficient associations (Rúa et al. 2016); 2) invasive species, usually associate with generalist widespread mycorrhizal fungi (Moora et al. 2011) establishing said associations with ease; and 3) Wagg et al. (2014) found that in mixed species assemblages, after manipulating microbial diversity levels, grasses seemed to be benefitted by low diversity microbial communities. Put together, this can explain, although not mechanistically, why both species produced more biomass with sterilized *U. eminii* legacies, which contained a small fraction of an inoculum. In this experiment we showed that a simpler microbiota is capable of stimulating higher biomass production than a putatively more diverse inoculum, in a monoculture. If proven, this relationship between lower microbe diversity and grass-biomass production could have direct implications for the restoration of heavily degraded areas, such as former mining sites; it would be advisable to initiate restoration of those areas with grasses. Benefits provided by low diversity inoculum for grasses should be investigated possibly affecting the way we understand grass physiology as well.

Urochloa eminii legacies and invaded inocula rendered high levels of root mycorrhizal colonization for both species, in line with evidence that *U. eminii* is a very efficient AMF propagator, effectively changing the soil microbiota; it is even used in laboratories for mycorrhizal spore propagation (Banuelos et al. 2013). Recent evidence also points to *U. eminii* ability to accumulate spores in invaded Cerrado areas (Leite et al. 2019); hence, any living soil in which *U. eminii* has grown should harbor high amounts of mycorrhizal spores, ultimately affecting future colonizers of this soil patch.

Diectomis fastigiata improved development with *U. eminii*-associated legacies and soil inocula could have been caused by an effect of higher availability of mycorrhizal spores, or even other microorganisms. Mycorrhizal colonization is capable of overcoming negative plant-soil feedbacks, *via* plant immune system stimulation (Wang et al. 2019; Cameron et al. 2013). Spore diversity can be an important factor positively influencing mycorrhizal colonization (Fitzsimons & Miller 2010) and high spore loads lead to elevated mycorrhizal colonization (Khakpour & Khara 2012). Ultimately, the origin of inoculated soil, can affect net plant response (Ma et al. 2018), as demonstrated here, directly affecting plants ability to recruit available microorganisms. We also acknowledge that although our results

point to a strong effect caused by mycorrhizal fungi, we cannot attest for sure observed effects are definitely caused by mycorrhizal fungi and not by other organisms present in the soil. Understanding the biological influences of inocula, and especially of mycorrhiza, in species used for restoration is necessary to better predict outcomes and influence the system in desired ways.

Legacy effects

Diectomis fastigiata had a broader range of responses regarding treatment combinations, especially with self-legacy. *Diectomis* legacies stimulated the production of lower LMA and higher SRL compared to *U. eminii* legacies, this however, had little effect in either species biomass production, although both traits are considered to be acquisitive. This indicates the responses in terms of biomass production cannot be attributed to variation in these traits alone, indicating, microorganisms probably exert more influence towards biomass production than variation in morphological traits alone. Other plant size traits measured were not good predictors of biomass investment, and therefore are not discussed in detail.

Regarding *D. fastigiata* grown with self-legacy and native inoculum, initial positive effects from soil inoculation in the first phase disappeared, and a self-negative feedback emerged. This effect could have been caused by a build-up of host-specific pathogens (Mills & Bever 1998), or other natural enemies (Kardol et al. 2007), as is expected that native inocula harbor more pathogenic organisms because native soils usually have higher plant diversity. Pathogens could even be targeting soil mycorrhiza (Bennett & Klironomos 2019) as we observed a considerable smaller mycorrhizal presence in this treatment. Kulmatiski et al. (2008) in a meta-analysis found that there is a trade-off between fast growth rates and enemy defenses for annual species. Pathogen build-up through one or more plant cycles could explain the observed negative feedback for the annual *D. fastigiata*. This is in accordance to initial expectations the annual *D. fastigiata* would be more impaired by its own legacy, although we punctuate that impairment depends on inoculum origin.

Urochloa eminii legacies were no different from *D. fastigiata* legacies in terms of biomass production for *U. eminii*; making it difficult to assume positive feedbacks were happening. We also acknowledge that given that species grew in warm weather during the first phase and cooler weather during the second, this likely affected overall biomass production, for all plants. That is to say, the absence of a noticeable positive feedback might

lie in external causes; hence, we consider *U. eminii* to present neutral-to-positive feedbacks, irrespective of soil legacy, with effects being strengthened by low diversity inocula. This is not in accordance with previous hypothesis that the invasive *U. eminii* would benefit itself; it is rather indifferent to inoculum origin.

Ecological considerations and applications in restoration

Soil chemistry indicates species interactions with soils leave some sort of signature in terms of soil characteristics, probably associated to resource-uptake strategies (Waring et al. 2015) and interactions with microbes. There seems to be a complex interplay between inoculation, legacies and species identity, but due to the circumstantial nature of the evidence provided here, generalizations are complicated, and further investigations are needed.

The combination of distinct inocula and legacies can result in different outcomes. These outcomes are directly associated to each species ability to recruit and interact with soil microorganisms. *Diectomis fastigiata*, as an annual species, is more sensitive to negative effects of different inoculum-legacy combinations than the invader *U. eminii*, as initially hypothesized, showing a wider range of variation in trait responses to different treatments. *Urochloa eminii* on the other hand, had little variation in functional traits, presenting overall a neutral-to-positive feedback. In the legacy phase of the experiment, we found that the species has a trade-off in investments on LMA and SRL with no associated effects in biomass production. This helps demonstrate that part of the reason *U. eminii* is a successful invader lies in its ability to tolerate differences in soil history and changes in its functional traits, presenting a satisfactory performance in a range of conditions, meaning it has a broad ecological niche. Although it is a widespread invasive species, this study indicates the soil changes posed by *U. eminii* do not cause any sort of deficit for *D. fastigiata*, they actually benefit the species. Future studies should test if *U. eminii* presents negative effects for combinations of multiple native species.

The negative self-feedback observed for *D. fastigiata*, indicates it would probably not dominate the system for the next growth cycle. We highly recommend divesting from using *D. fastigiata* in ecological restoration, owing to the uncertainty regarding the species origin (Filgueiras 1988) as well as its self-negative feedback. We urgently need to invest in research on perennial grass species which have more chances of presenting no negative self-

legacy (Koziol et al. 2018) and are more likely to be benefitted by inoculation (Middleton and Bever 2010), improving restoration efforts. Our study shows the importance of considering plant-soil feedbacks when planning for restoration of native systems.

The effect of *D. fastigiata* legacies inducing higher SRL indicates there is a window of possibility to use plant legacies in restoration efforts, as suggested by Brinkman et al. (2017) for fen meadows. It can be possible to target desired functional traits of a restored community, planning restoration to encompass species succession or substitutions in order to fully explore the potential of legacy effects and inocula in restoration. In this case for example, *D. fastigiata* stimulated higher SRL, and potentially even more when inoculated with native soil, stimulating water and nutrient uptake (Pérez-Harguindeguy et al. 2003), these are likely to induce higher growth rates for coming species. It would be necessary therefore, to define goals of a desired targeted restored ecosystem in terms of functional traits, species composition and community response to foster ecosystem restoration using soil legacies and inoculation. Important to note, as of yet, there are no evidences if species succession occurs in the Cerrado grasslands because the vast majority of native species recruit via vegetative growth (Pilon et al. 2019; Silveira et al. 2020). Thus, restoration efforts in the Cerrado should not rely on the expectation that succession will occur in this ecosystem. Studies regarding vegetation dynamics of Cerrado communities are deeply needed and welcomed.

Future directions

Our experimental design was limited to the use of two grass species only. Mixed plant species assemblages should be used for both legacy and inoculation experiments, especially focusing on slow-growing species, to assess how multiple interactions between plants and microbes are shaped in complex settings. Future studies need to address the questions if and why some grass species are benefitted by lower diversity microbial communities. Finally, it is important that upcoming studies try to identify specifically which groups of organisms are being affected by the plants studied, so that more precise assertions can be made regarding soil legacies and plant-soil feed-backs. New studies targeting both natural vegetation and restored ecosystems dynamics, encompassing legacy and microbial effects are highly advised.

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Tables and Figures

Table 1. Survival of individuals (in percentage) used for statistical analysis according to each treatment, in the legacy phase. The species line, corresponds to which species grew in the legacy phase of the experiment, the legacy line indicates which species left a legacy in the pot, inoculum corresponds to the inoculum added to the soil in the conditioning phase: **I** corresponds to invaded-soil inoculum, **N** corresponds to native-soil inoculum and **S** corresponds to sterilized-soil inoculum. Each treatment consisted of 8 pots and two individuals per pot, to a maximum of 16 individuals per treatment.

Species	***Diectomis fastigiata***						***Urochloa eminii***					
Legacy	*D. fastigiata*			*U. eminii*			*U. eminii*			*D. fastigiata*		
Inoculum	**I**	**N**	**S**	**I**	**N**	**S**	**I**	**N**	**S**	**I**	**N**	**S**
Survival	81.25%	62.5%	18.75%	87.5%	93.75%	81.25%	100%	87.5%	87.5%	100%	68.75%	93.75%

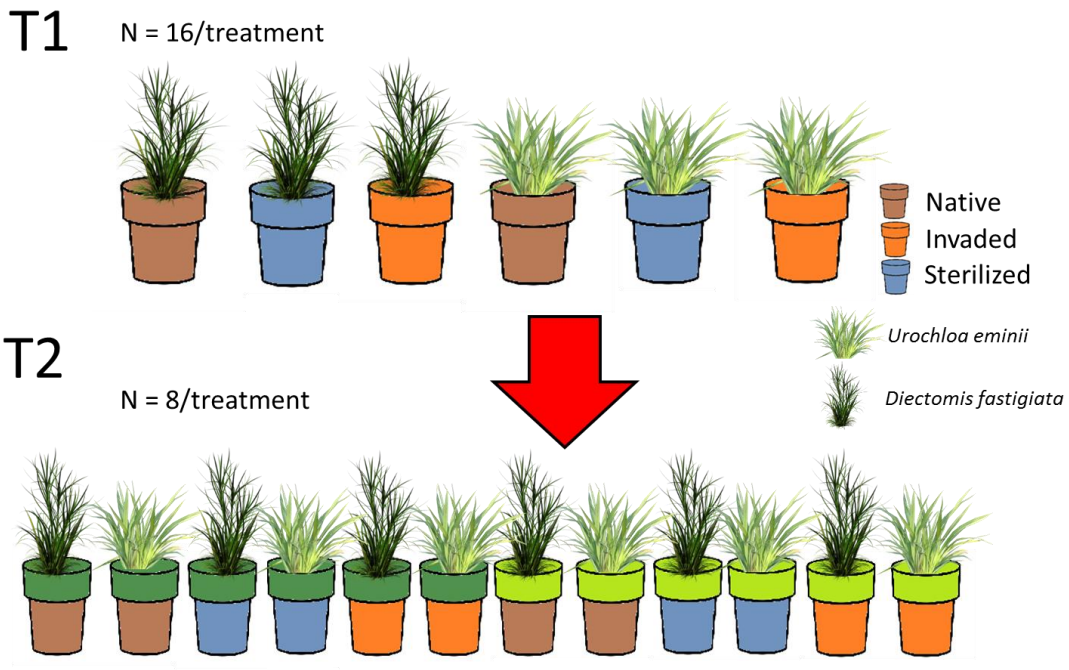

Figure 1. Schematic design of the experiment. T1 represents the first phase of the experiment and T2 the second phase. In T1 pot color represents soil inoculum origin in T1. In T2 the color in the base of the pot represents previous inoculum whereas the color in the top of the pot represents previous legacy, dark green: *D. fastigiata* legacies and light green: *U. eminii* legacies.

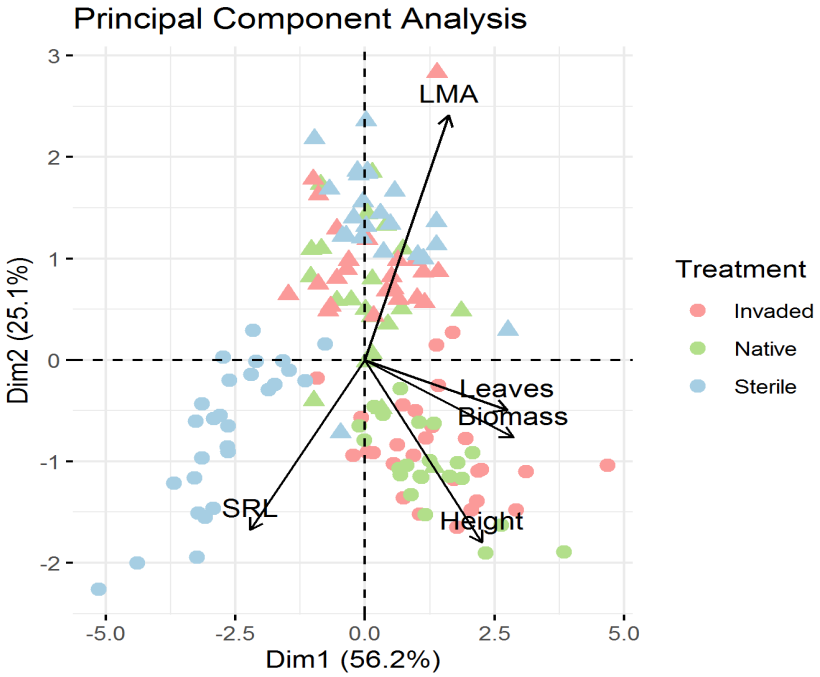

Figure 2. PCA with plant functional traits and biomass from the conditioning phase of the experiment. Triangles represent *Urochloa eminii* and circles represent *Diectomis fastigiata*. Colors indicate inoculum origin.

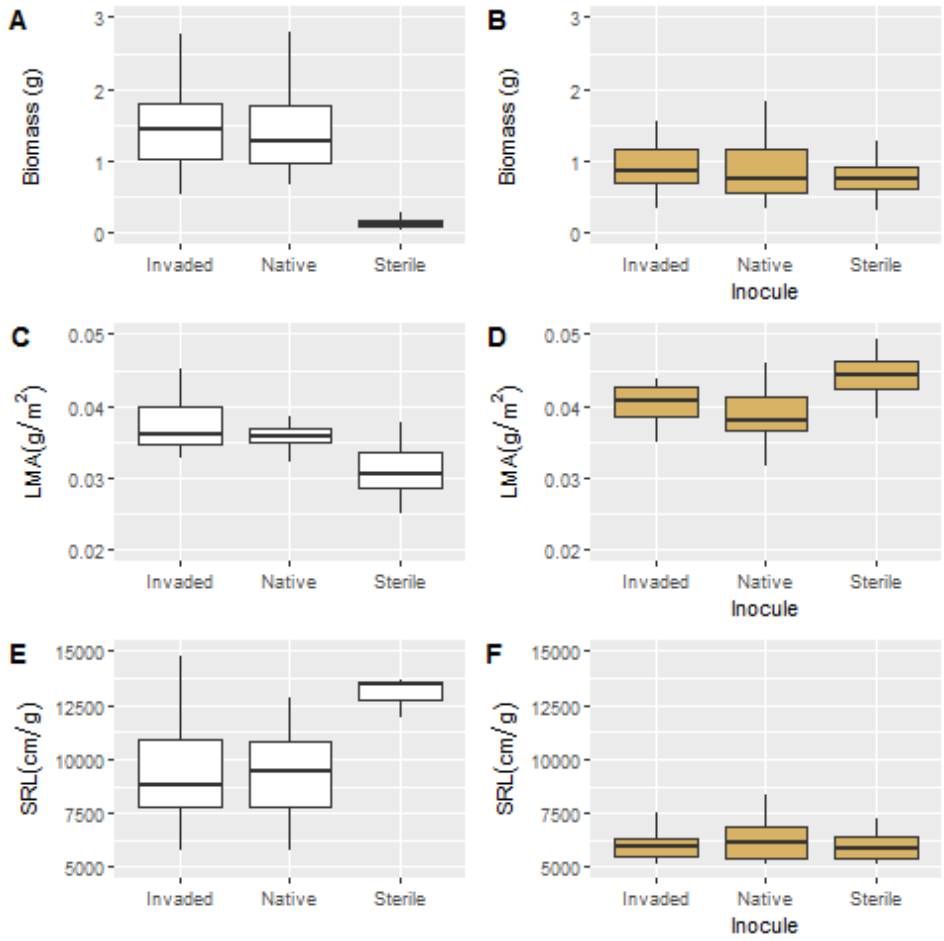

Figure 3. Aboveground biomass and functional traits of *D. fastigiata* (white; left) and *U. eminii* (orange; right) grown in the conditioning phase in sterilized soil with different soil inocula: Aboveground biomass (A, B); leaf mass per area (LMA) (C, D), specific root length (SRL) (E, F).

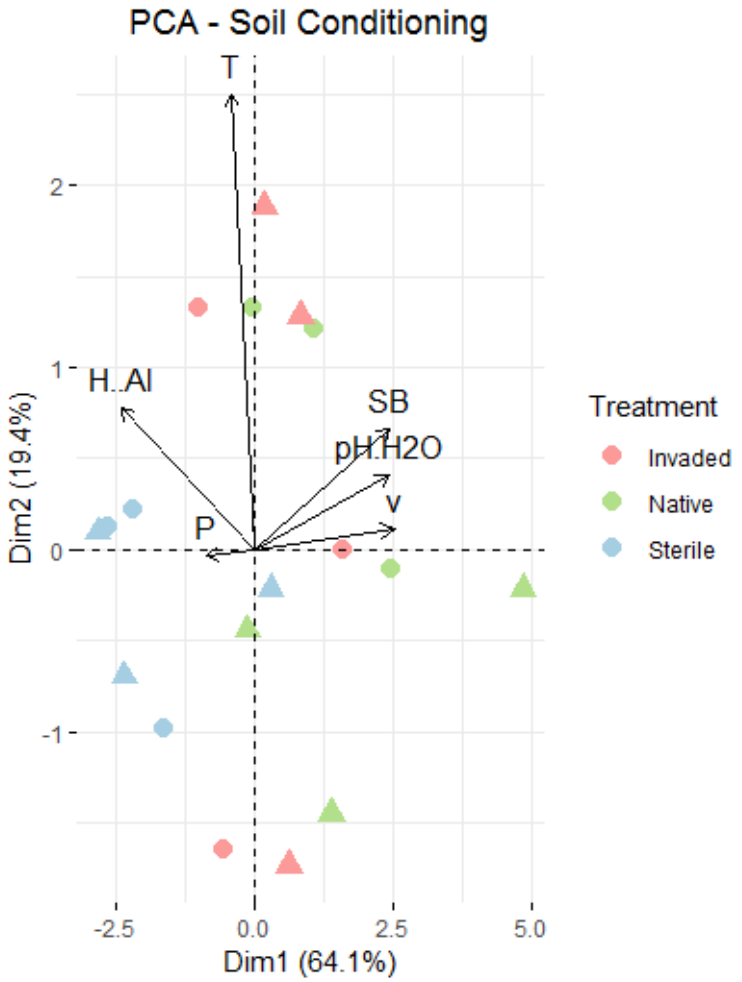

Figure 4. Principal component analysis from after the conditioning phase. Each point represents a sample, triangles represent *U. eminii* soils and circles represent *D. fastigiata* soils. Colors indicate which inoculum was used. T = cation exchange capacity, SB = sum of bases, pH.H2O = pH measured in water, v = base saturation, P = phosphorus, H.Al = potential acidity.

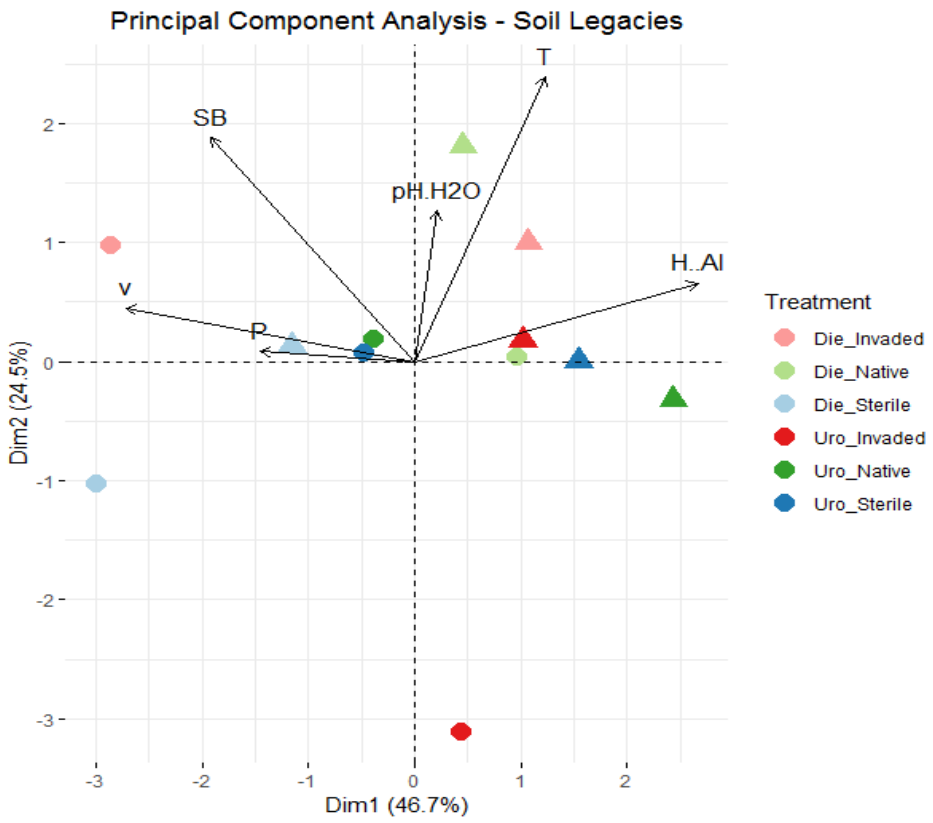

Figure 5. Principal Component Analysis of soils from after the legacy phase. Samples were compound containing soils from three pots. Point shapes represent the species grown in the legacy phase, circles represent *D. fastigiata* and triangles represent *U. eminii*, colors indicate which plant grew in the conditioning phase (soil legacy) combined with the soil inoculum, lighter colors with the prefix 'Die_' indicate *D. fastigiata* legacies whereas darker colors with the prefix 'Uro'indicate *U. eminii* legacies. pH.H2O = pH measured in water, T = cation exchange capacity, H.Al = potential acidity, P = phosphorus, v = base saturation, SB = sum of bases.

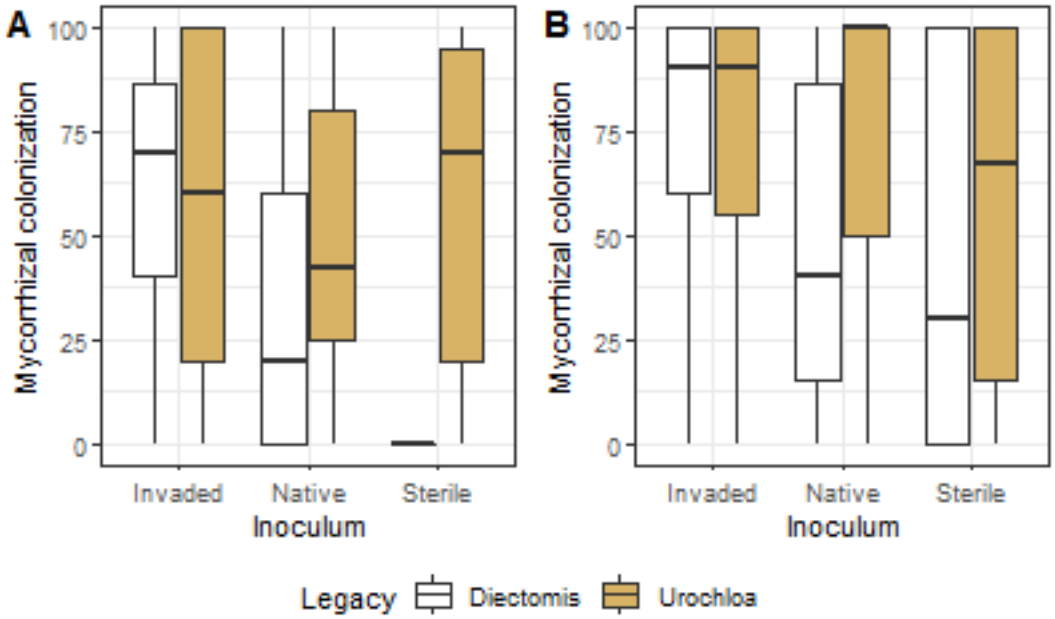

Figure 6. Mycorrhizal colonization for the legacy phase of the experiment. On the left side are presented results for *Diectomis fastigiata* and on the right for *Urochloa eminii*. White boxes represent *D. fastigiata* legacies and orange boxes *U. eminii*.

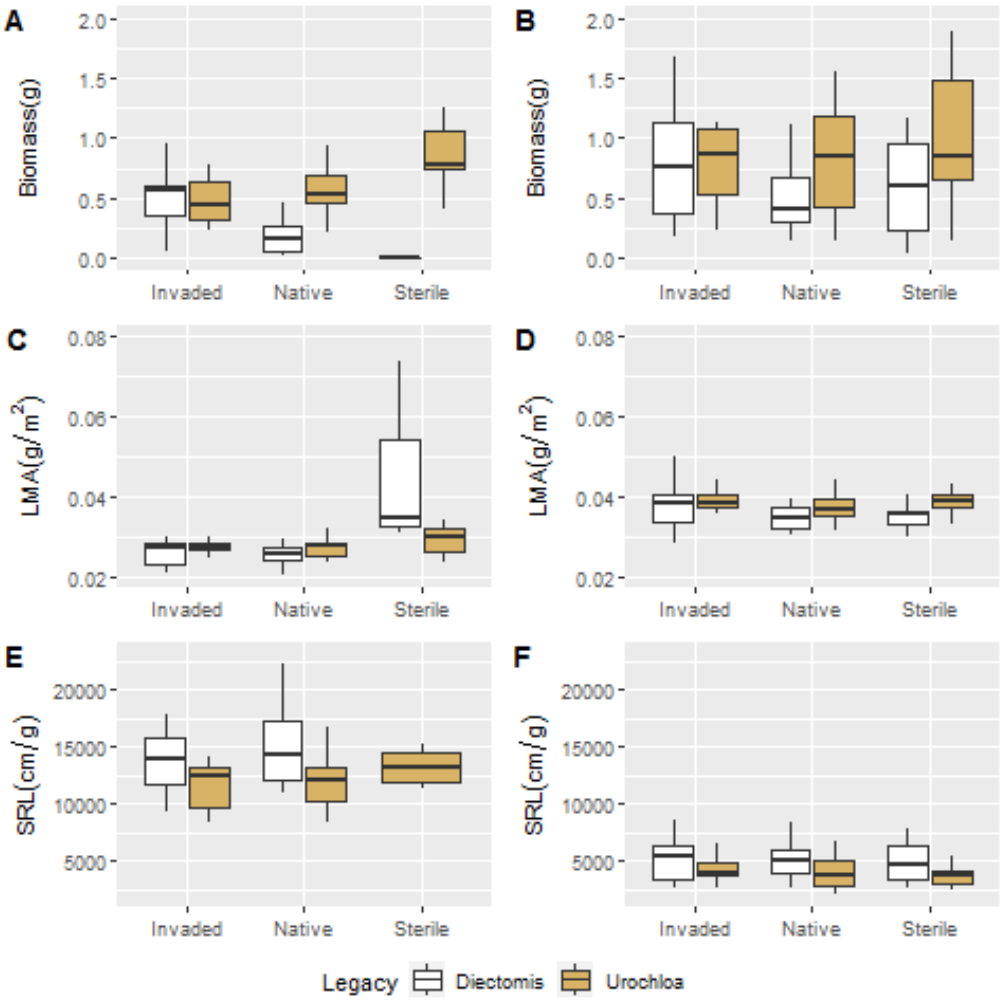

Figure 7. Aboveground biomass and functional traits for *D. Fastigiata* and *U. eminii* during the legacy

phase of the experiment. On the left side results are presented for *D. fastigiata* and on the right side for *U. eminii*. Legacies are represented by box color, white corresponding to *D. fastigiata* legacies and orange to *U. eminii*. On the x axis labels we can see the inoculum received during the conditioning phase. A and B correspond to aboveground biomass, C and D to leaf mass per area (LMA) and E and F to specific root length. *SRL in graph E does not show for *D. fastigiata* legacies because values were way out of range compared to others, starting well above 100.000 cm/g.

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